A Transmissible Interfering Component of Vesicular Stomatitis Virus Preparations

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SUMMARY: Serial (more than two) undiluted passages of vesicular stomatitis virus in chick embryo cell monolayers gave low virus yields (e.g. 10 plaque-forming units (pfu)/cell) compared with dilute passage (e.g. 1000 pfu/cell). It was concluded that the low yields resulted from production of a transmissible interfering component since a mixture of undiluted passage with dilute passage virus stocks also gave low yields. Lack of infection of some cells, lack of time for complete growth, genotypic viral change, virus inactivation, poor cultures or media variations were excluded as explanations. Yield increase with inoculum dilution was marked with undiluted passage but not with dilute passage stocks. Multiple infection with live virus (dilute passage stocks) gave high yields, and mixed infection between plaque-forming units and transmissible interfering component (T) was required for interference and for propagation of T. Heat or u.v.-inactivation of live virus did not render it able to interfere (i.e. did not form T). The interference was mostly exclusion (all-or-none) with some slowing of release, since cells which released plaque-forming units released the normal number. T by itself was not toxic, and those cells which received T but not plaque-forming units in the first cycle of growth were protected from degeneration for several days. Cells which received T and plaque-forming units were killed whether or not they released virus.

Routine preparation of vesicular stomatitis virus stocks from chick embryo cell monolayers (Cooper, 1957a) sometimes gave apparent virus yields of less than 10 plaque-forming units (pfu)/cell (assuming that all cells in the monolayer released virus), while at other times the apparent yield was 1000 pfu/cell or more. A cytopathic effect was always marked or severe in all cells, when sufficient inoculum virus or time of growth was allowed for all cells to complete their virus release before harvesting. Poor culture conditions, media variations or inactivation of extracellular virus did not account for the low yields.

An incomplete form of the virus might have been responsible, since undiluted or low-dilution tissue-culture fluids were often used as inocula, although one or two undiluted passages usually gave high yields, and yields were in general unaffected by inoculum dilution (Cooper, 1958a). However, these experiments all used high-yield dilute passage stocks, since all low-yield stocks were rejected for experimental purposes. A study of the derivation of both Indiana and New Jersey serotype stocks revealed that low-yield preparations always arose by several serial low-dilution passages of initially high yield dilute passage stocks; this has now been frequently repeated. The present paper demonstrates that vesicular stomatitis virus, although not regarded as

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a myxovirus, may yet develop a serially transmissible but non-plaque-forming component which interferes with homologous virus growth; despite some analogies with the incomplete form of influenza virus, however, it cannot yet be regarded as an incomplete form of vesicular stomatitis virus. This is discussed by Bellett & Cooper (1959).

METHODS

Preparation and storage of virus stocks and assays were as described by Cooper (1957a, 1958a). All assays and virus growth were made in 9 cm. diam. Petri dish monolayers containing $2 \pm 0.5 \times 10^7$ chick embryo cells. Unless otherwise specified, the media used were as previously described except that the Earle's saline (ES) + 5% (v/v) horse serum used for cell and virus growth was supplemented with 5 mg. lactalbumin hydrolysate/ml. and 1 mg. Difco yeast extract/ml. This supplemented Earle's saline is meant where 'medium' is subsequently mentioned without qualification. Adsorption of virus by monolayers for virus growth (but not assays) was carried out in the incubator hood described by Cooper (1958b); cells were resuspended for assay of infective centres as described in the same paper. Stocks are numbered according to serotype and type of passage, e.g. NJ.UP. 6, IND.DP. 25.

RESULTS

Effect of serial dilute and undiluted passage on virus yield/cell

Table 1 gives an abstract from the derivation of some fifty virus stocks of Indiana serotype used in experiments described here and elsewhere (Cooper, 1958a, b) and made in London in 1956-7. About twenty-five New Jersey stocks made at the same time, and twelve made in California in 1954-5 and used by Cooper (1957a, b, c; 1958a), had a similar derivation. Despite considerable individual variations in yield, one or two undiluted passages from a high-titre stock rarely gave low titres (particularly when of very dilute passage origin), but serial passage undiluted or at low dilution markedly lowered titres. Titres became high again on dilute passage.

Serial undiluted passage depressed New Jersey more than Indiana titres; one Indiana (Pl. 2, fig. 4) and three New Jersey undiluted passages showed less or no cytopathic effect after prolonged incubation despite the presence of adequate numbers of plaque-forming units. The controls were healthy microscopically, and simultaneous undiluted passage with dilute passage stocks gave good cytopathic effects. The titres were still those to be expected from unadsorbed inoculum, despite considerable thermal inactivation (3 days at 37°). The illustration of cell protection by a low-titred Indiana undiluted passage stock in Pl. 2, fig. 4, is compared with uninfected cells (Pl. 1, figs. 1, 2) and cells infected with an Indiana dilute passage stock (Pl. 2, fig. 3).

Unlike New Jersey virus, complete loss of Indiana virus cytopathic effect was not found on serial undiluted passage. Instead, undiluted passage yields stabilized at about 10% of the dilute passage yields with occasional increases suggesting reversion (e.g. UP. 40, Table 1). One stock (IND.UP. 15) gave a
Table 1. Abstract of genealogy of vesicular stomatitis virus stocks (Indiana serotype)

Prepared by 30 min. adsorption of 0.5 mL inocula by monolayers containing $2 \pm 0.5 \times 10^6$ cells and overlaid by 5 mL of medium. All monolayers inoculated on one date are comparable. The media were removed after 22 hr. and frozen for virus assay. Cytopathic effect was always very marked or complete while simultaneous controls were healthy. UP = undiluted passage, DP = dilute passage, the number being the stock number and the fraction being the dilution factor. The number in brackets = harvested yield in pfu/cell; '1 pfu' indicates stock prepared from a single picked plaque. 'u.v. treated' = u.v. irradiated to 0.17 % survivors, 'heated' (10 min. at 50°) to 2-1 % survivors, then both were plated undiluted with sufficient live UP. 22 virus (3 %, v/v) to infect all cells in the first cycle.

<table>
<thead>
<tr>
<th>Date on which culture was inoculated</th>
<th>UP. 15 (12)</th>
<th>UP. 22 (550)</th>
<th>UP. 17 (800)</th>
<th>UP. 34 (14)</th>
<th>u.v. treated</th>
<th>heated</th>
</tr>
</thead>
<tbody>
<tr>
<td>16. i. 57</td>
<td>10^-3</td>
<td>10^-3</td>
<td>10^-3</td>
<td>10^-3</td>
<td>10^-3</td>
<td></td>
</tr>
<tr>
<td>17. i. 57</td>
<td>UP. 26 (500)</td>
<td>DP. 17 (800)</td>
<td>UP. 25 (500)</td>
<td>DP. 16 (1060)</td>
<td>DP. 12 (1500)</td>
<td>UP. 18 (800)</td>
</tr>
<tr>
<td>17. i. 57</td>
<td>10^-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22. i. 57</td>
<td>UP. 30 (15)</td>
<td>UP. 28 (50)</td>
<td>UP. 29 (400)</td>
<td>UP. 35 (275)</td>
<td>UP. 42 (500)</td>
<td>DP. 21 (1250)</td>
</tr>
<tr>
<td>23. i. 57</td>
<td>10^-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24. i. 57</td>
<td>UP. 32 (50)</td>
<td>UP. 31 (175)</td>
<td>UP. 36 (250)</td>
<td>UP. 45 (300)</td>
<td>UP. 40 (250)</td>
<td></td>
</tr>
</tbody>
</table>
high-titre undiluted passage yield and the same yield on dilute passage (Table 4) strongly suggesting reversion; it was grown at 32°, but this experiment was not repeated.

When the dilute passage and undiluted passage stock titres are grouped without considering their parentage in more detail, the mean titres in pfu/ml. are: New Jersey, $1.7 \times 10^9$ (dilute passage) $1.3 \times 10^8$ (undiluted passage); Indiana, $2.7 \times 10^9$ (dilute passage) and $6 \times 10^8$ (undiluted passage). Thus mean undiluted passage and dilute passage titres differed significantly; since many ‘dilute’ passages were at low dilution, often from an undiluted passage parent, and some undiluted passage stocks were directly derived from high-titre dilute passage stocks, this significance is enhanced by the need for several serial undiluted passages before the yield was greatly lowered. In fact, if there were an equal number of alternating dilute and undiluted passages one might see no difference.

**Factors other than interference which may contribute to a low yield**

The existence of interference is postulated later; meanwhile the preceding data, which show low yields with some inocula but not with others, are not significant without considering the system used.

**Culture variations.** The culture method used (Cooper, 1957a) usually gave a nearly confluent monolayer with cells partly spread after 20 hr. (Pl. 1, fig. 1), becoming fully confluent by 46 hr. (Pl. 1, fig. 2) by cell spreading and enlargement rather than by cell division (Cooper, 1957b). The amount of debris was minimal; $1-2 \times 10^7$ cells were resuspended by trypsin and the microscopical appearance of the cultures was very reproducible. Some non-confluent batches, or individual ‘toxic’ monolayers, were discarded. Except with very dilute virus inocula, the cytopathic effect at 20–24 hr. after inoculation was always extensive in all cells whether with undiluted or dilute inocula, undiluted or dilute passage stocks or with high or low yields (Pl. 2, fig. 3). Four exceptions to a complete cytopathic effect have been mentioned, and were due to interference. Table 1 compares dilute and undiluted passages in single batches of monolayers, and shows the dependence of yield on the history of the stock.

Growth curve studies (Cooper, 1957a, b, c; 1958a) showed that monolayers of one batch released virus very similarly, although some batch-to-batch variation occurred. It is concluded, therefore, that variations in the cultures used did not account for the low yields obtained with certain inocula.

**Effect of different media.** Apparently identical well-spread monolayers were formed in the medium generally used or in medium ‘199’ (Morgan, Morton & Parker, 1950)+5% (v/v) rabbit or horse serum or chick embryo extract. Any of these media, used for monolayer formation or virus release, allowed undiluted passage yields characteristic of stock history and independent of medium.

**Inactivation of extracellular virus.** The heat lability of vesicular stomatitis virus (half-life 1–2 hr. at 37° for both serotypes; Fig. 1) will decrease the apparent yields between release and harvesting, but when undiluted passage and dilute passage stocks were compared with the same opportunities for
Transmissible interfering component

thermal inactivation (same multiplicity of infection) the yields were still low and high, respectively. There was no evidence for an increased heat lability on serial undiluted passage, as a permanent change is excluded by obtaining high yields again on one dilute passage (single plaque). Figure 1 shows no marked phenotypic difference between the stabilities of dilute passage and undiluted passage infective components.

Interference by an accumulation of thermally inactivated virus in the stocks

Fig. 1. The stability of vesicular stomatitis virus in medium (supplemented Earle's saline plus 15 % (v/v) heat-inactivated horse serum) at 37°. Stocks were diluted to about 500 pfu/ml. in warm medium, and samples were taken at intervals and assayed at once for infectivity. The T-containing stocks IND.DP. 6 (●) and N.J.UP. 7 (▲) were compared with relatively T-free stocks IND.UP. 22 (○) and N.J.DP. 10 (△).

Table 2. The stability of vesicular stomatitis virus in buffer at various pH values and in other media

The buffer solutions (m-citrate in m-phosphate) were adjusted to various pH values with a pH meter and HCI, and autoclaved. A small amount (0·1 ml.) of a New Jersey serotype virus stock of known titre was added to 4 ml. medium to give 1·5 × 10⁵ pfu/ml., and the suspensions were stored at 0° for 21 hr. when they were assayed at once.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Infectivity remaining after 21 hr. at 0°. (Percentage of original)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer pH 9·1</td>
<td>33</td>
</tr>
<tr>
<td>8·0</td>
<td>27</td>
</tr>
<tr>
<td>7·1</td>
<td>29</td>
</tr>
<tr>
<td>5·8</td>
<td>29</td>
</tr>
<tr>
<td>4·9</td>
<td>11</td>
</tr>
<tr>
<td>4·1</td>
<td>3·5</td>
</tr>
<tr>
<td>3·0</td>
<td>c. 0·1</td>
</tr>
<tr>
<td>2·0</td>
<td>&lt; 0·1</td>
</tr>
<tr>
<td>0·25 m-sucrose</td>
<td>3·9</td>
</tr>
<tr>
<td>0·88 m-sucrose</td>
<td>&lt; 0·1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>29</td>
</tr>
<tr>
<td>Earle's saline</td>
<td>49</td>
</tr>
</tbody>
</table>
is not an adequate explanation, since washing the cells after infection during serial undiluted passage did not increase the yield; virus killed by heat, storage at —20° or u.v. irradiation does not interfere homotypically (Cooper, 1958a) and did not depress yield when added deliberately (Table 4). Small changes in pH value or osmotic pressure did not appear to affect infectivity, since the rate of loss of infectivity by vesicular stomatitis virus is the same between pH 6 and 9, although much higher below pH 5 (Table 2); the half-life was similar in distilled water, buffer or Earle's saline (about 10 hr. at 0°), although much less in sucrose solutions. Undiluted passage gave low yields from undiluted passage stocks and high yields from dilute passage stocks whether or not the serum had previously been heated at 56° for 80 min. No antibodies fixing complement with undiluted virus stocks were found by Brooksby's (1948) method in the horse serum used, although complement fixation was very good with specific guinea-pig immune sera. Thus, low yields from some inocula but not others cannot be accounted for by extracellular inactivation of virus.

**Increasing yield with increasing inoculum dilution with undiluted but not dilute passage stocks**

In a number of experiments, simultaneous comparison of undiluted and dilute passage yields with the same undiluted passage stock and batch of monolayers showed that dilute inocula gave higher yields than the same preparation inoculated undiluted, similar to the effect described by von Magnus (1951) for influenza virus. No difference in yield between undiluted passage or a wide range of dilute passages of New Jersey dilute passage stocks of high titre had previously been found (Cooper, 1957a, 1958a). This suggested that, like influenza virus, the effect of inoculum dilution on yield is more marked with undiluted than with dilute passage stocks. Tables 5-6 confirm the difference between such stocks in the effect on yield of inoculum dilution for both vesicular stomatitis serotypes. Each table represents one experiment with one batch of monolayers; subsequent assays were also performed with a single batch of monolayers.

**IND.UP. 22** as dilute passage stock (high titre, one undiluted passage from a high titred dilute passage stock and giving high titres on further undiluted passage) was compared with **IND.DP. 6** as undiluted passage stock (low titre, from a long undiluted + low dilution passage series, and giving low titres on further undiluted passage (Tables 3 and 4)). The paradox in nomenclature emphasizes the importance of serial rather than a single undiluted passage (for **IND.UP. 22**) in inducing this interference, and the inefficacy of one low-dilution passage (for **IND.DP. 6**) in removing it. **IND.UP. 22** showed perhaps a little, while **IND.DP. 6** showed a marked, decrease in yield at the two highest concentrations.

The lack of dilution effect with dilute passage New Jersey stocks is shown in Table 5. Table 6 compares two stocks of an undiluted passage series, **NJ.DP. 8** (of high titre and giving a fairly high titre on one undiluted passage but derived by one very low dilution (1:2) passage from the original stock) and **NJ.UP. 12** (of fairly high titre but giving very low titres on subsequent undiluted passage...
and derived from NJ.DP. 8 by one undiluted passage. Both NJ.DP. 8 and NJ.UP. 12 gave marked dilution effects, but the effect with the later undiluted passage stock was greater than with the earlier, especially at the highest concentrations.

Table 3. The effect of inoculum dilution on 24 hr. yields from a serial low-dilution passage Indiana serotype vesicular stomatitis virus stock (IND.DP.6, 8·6 x 10⁷ pfu/ml.) using monolayers of one batch

The 0·5 ml. inocula were removed after 1 hr. in the incubator hood, and 5 ml. of warm medium were added for virus release at 37°. M = input multiplicity of infection; cytopathic effect was severe in all cells at harvest.

<table>
<thead>
<tr>
<th>Inoculum dilution</th>
<th>Yield</th>
<th>M</th>
<th>pfu/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>1</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>1/8</td>
<td>0·25</td>
<td>315</td>
<td></td>
</tr>
<tr>
<td>1/16</td>
<td>0·13</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>1/32</td>
<td>0·06</td>
<td>248</td>
<td></td>
</tr>
<tr>
<td>1/320</td>
<td>0·006</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. The slight effect of inoculum dilution on 20 hr. yield from certain Indiana serotype vesicular stomatitis virus stocks

The following stocks were compared: a dilute passage stock after one undiluted passage (IND. UP. 22); a serial undiluted passage stock which has reverted to non-auto interference (IND.UP. 15); IND.UP. 22 stocks containing a large excess of either ultraviolet- or heat-inactivated Indiana virus. For the latter, some IND.UP. 22 stock was u.v. irradiated to 0·17% survivors, and some more heated to 2·1% survivors (10 min. at 50°); sufficient untreated IND.UP. 22 was re-added (3%, v/v) to infect all cells in the first cycle on undiluted passage. The inocula (0·5 ml.) were removed from monolayers after 1 hr. in the incubator hood, which were washed once with PBS and then given 5 ml. of warm medium for virus release at 37°; all monolayers showed complete or severe cytopathic effect at 20 hr. M = multiplicity of infection of live virus; the multiplicity of u.v.- and heat-inactivated virus will be the same as that of live IND.UP. 22.

<table>
<thead>
<tr>
<th>Inoculum dilution</th>
<th>Yield</th>
<th>u.v. treated</th>
<th>Heated</th>
<th>IND.UP 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>pfu/cell</td>
<td>M</td>
<td>pfu/cell</td>
</tr>
<tr>
<td>Undiluted</td>
<td>50</td>
<td>500</td>
<td>2</td>
<td>960</td>
</tr>
<tr>
<td>1/2</td>
<td>25</td>
<td>660</td>
<td>1</td>
<td>670</td>
</tr>
<tr>
<td>1/4</td>
<td>12</td>
<td>840</td>
<td>0·5</td>
<td>1000</td>
</tr>
<tr>
<td>1/10</td>
<td>5</td>
<td>1250</td>
<td>0·2</td>
<td>1110</td>
</tr>
<tr>
<td>1/20</td>
<td>2·5</td>
<td>1000</td>
<td>0·1</td>
<td>1000</td>
</tr>
<tr>
<td>1/100</td>
<td>0·5</td>
<td>1250</td>
<td>0·02</td>
<td>1500</td>
</tr>
<tr>
<td>1/1000</td>
<td>0·05</td>
<td>1060</td>
<td>0·002</td>
<td>1500</td>
</tr>
</tbody>
</table>

The yield on undiluted passage of dilute passage stocks in presence of u.v.- and heat-inactivated virus, and at 41°

Addition of u.v.- and heat-inactivated dilute passage stocks to the same untreated dilute passage stock (Table 4) did not cause any depression of yield on one undiluted passage, and further undiluted passage (Table 1) did not suggest that much interfering component had been produced on this passage.
Table 1 shows that, whereas one undiluted passage at 41°C gave a low yield, further undiluted passage of this low yield stock gave high yields indicating that there had been no accumulation of the interfering component during growth at the higher temperature. It would seem therefore that the interfering component differs fundamentally from heat- or u.v.-inactivated virus.

Table 5. Lack of effect of dilution on 10 hr. yield of a dilute passage New Jersey serotype vesicular stomatitis virus stock (9-2 × 10<sup>8</sup> pfu/ml)

Inocula (0-5 ml.) were adsorbed for 30 min., the monolayers washed twice with medium and overlaid with 5 ml. warm medium for virus release at 37°C.

<table>
<thead>
<tr>
<th>Inoculum dilution</th>
<th>Multiplicity of infection (pfu/cell)</th>
<th>10 hr. yield (pfu/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>25</td>
<td>180</td>
</tr>
<tr>
<td>1/2</td>
<td>12</td>
<td>220</td>
</tr>
<tr>
<td>1/5</td>
<td>4-5</td>
<td>200</td>
</tr>
<tr>
<td>1/10</td>
<td>2-3</td>
<td>220</td>
</tr>
<tr>
<td>1/20</td>
<td>1-2</td>
<td>180</td>
</tr>
<tr>
<td>1/50</td>
<td>0-45</td>
<td>200</td>
</tr>
<tr>
<td>1/100</td>
<td>0-23</td>
<td>220</td>
</tr>
<tr>
<td>1/200</td>
<td>0-12</td>
<td>115</td>
</tr>
</tbody>
</table>

Table 6. The effect of inoculum dilution on 22 hr. yields from two serial low-dilution New Jersey serotype vesicular stomatitis virus stocks, compared using one batch of monolayers

NJ.UP. 12 was derived from NJ.DP. 8 by one undiluted passage. The 0-5 ml. inocula were removed after 1 hr. in the incubator hood, monolayers were washed once with 5 ml. PBS, and 5 ml. warm medium added for virus release at 37°C. M = input multiplicity of infection of live virus. Cytopathic effect was severe in all cells at harvest.

<table>
<thead>
<tr>
<th>Inoculum dilution</th>
<th>Yield M (pfu/cell)</th>
<th>Yield M (pfu/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>60</td>
<td>11</td>
</tr>
<tr>
<td>1/3</td>
<td>20</td>
<td>c. 20</td>
</tr>
<tr>
<td>1/10</td>
<td>6</td>
<td>c. 30</td>
</tr>
<tr>
<td>1/30</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>1/100</td>
<td>0-6</td>
<td>55</td>
</tr>
<tr>
<td>1/300</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1/1,000</td>
<td>0-06</td>
<td>65</td>
</tr>
<tr>
<td>1/10,000</td>
<td>0-006</td>
<td>180</td>
</tr>
<tr>
<td>1/100,000</td>
<td>0-0006</td>
<td>100</td>
</tr>
</tbody>
</table>

Comparative one-step virus release from monolayers infected with dilute or undiluted passage stocks

A comparison of one-step virus release was made between (a) cells completely infected (multiplicity = 6) with dilute passage stocks, and (b) cells with the same multiplicity of infective virus but also with a high multiplicity of the interfering component T, which is discussed below.

Figure 2 and Table 7 show one of three such experiments. A high proportion (about 80%) of the cells which had received infective virus had nevertheless...
Transmissible interfering component

been prevented from releasing infective progeny, so that infective virus was presumed to be 'excluded' by some component of the undiluted passage stock. The other experiments, summarized in Table 8, show a similar degree of exclusion, and in all three experiments those cells able to release virus did so to an approximately normal yield (84-178 % of controls), as the total yield of the culture was reduced by about the same factor as the number of cells able to release virus. Thus the lowered yield of the undiluted passage

Table 7. Prevention of virus release by the T component

Of a batch of 20 monolayers of 1.5 x 10^7 cells, one group of 10 received a dilute passage stock as inoculum (IND.DP. 18, 2.7 x 10^6 pfu/ml. diluted 1/10 in medium to give a multiplicity of infection of 10), and the other group of 10 received an undiluted passage stock (IND.DP. 18 diluted 1/10 in IND.UP. 11, < 5 x 10^7 pfu/ml., giving an infective multiplicity = 10 but with additionally an excess of T). After 1 hr. adsorption at 37°, while still in the incubator hood the inoculum was removed, cells washed once with warm PBS, and 5 ml. warm medium were added to each monolayer. After 3 hr. infection, cells from 2 monolayers of each group were trypsinized into 1 ml. PBS for infective centre assays; the table refers to the composition of this suspension. The supernatant medium was removed from the remaining monolayers, still at 37°, at intervals for virus assay during 1-step growth (see Fig. 2). A and B (col. 1) are duplicates.

<table>
<thead>
<tr>
<th>Infective centres per monolayer</th>
<th>Not sedimented by 8 min. at 1000 rev./min.</th>
<th>Total cells recovered per monolayer</th>
<th>Plating efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolayer</td>
<td>(1)</td>
<td>(2)</td>
<td>(3)</td>
</tr>
<tr>
<td>Dilute passage inoculum</td>
<td>A</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Undiluted passage inoculum</td>
<td>B</td>
<td>0.84</td>
<td>6 x 10^6</td>
</tr>
</tbody>
</table>

Table 8. Comparison of the plating efficiencies of the infected cell suspensions with final yields during one-step growth of virus from dilute passage (DP) and undiluted passage (UP) stocks

Figure 2 and Table 7 describe expt. 3, and expts. 1 and 2 are duplicates of 3. Plating efficiency = infective centres/ml. divided by total cells/ml.
stock is accounted for almost entirely by this exclusion, so that the excluding component is also the yield-reducing component. The latent period of the yielding cells in the interfered cultures was normal in length, although the rate of release was somewhat slowed. Despite the complete prevention of release from most of the cells, cytopathic effect was severe in all cells at 22 hr.
as in other undiluted passage cultures.

![Graph showing virus release](image)

**Fig. 2.** The effect of the T component on one-step vesicular stomatitis virus release from identical chick embryo cell monolayers. Half of the monolayers received dilute passage virus sufficient to infect all the cells (○), the remainder received the same multiplicity of dilute passage virus with additionally a high multiplicity of T (●); full experimental details are given in Table 7. The supernatant fluid was removed from one monolayer of each group after various intervals at 37°C and frozen pending assay of pfu. The point on the ordinate represents infective virus initially added, and the broken horizontal lines the number of cells/ml able to release virus, corrected for plating efficiency (see Table 7) to give a truer estimate of latent period.

**DISCUSSION**

As compared with serial dilute passage, serial undiluted passage of dilute passage vesicular stomatitis virus stocks of either serotype (New Jersey or Indiana) gave low yields of infective virus, unaccounted for by variations in media, cultures or heat labilities. As a single dilute passage of the low-yielding stock, sometimes from a single plaque, gave high yields again, there must have been no genotypic change in the majority of the virus particles. Multiple adsorption of infective virus alone (dilute passage inocula) did not give low yields, but yields increased with dilution of undiluted passage.
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stocks. In some cultures apparent protection of nearly all cells through many viral growth cycles, with no evidence of cytopathic effect and very low yields, resulted from low-titre undiluted passage stocks containing insufficient infective virus to infect all cells initially. A mixture of dilute and undiluted passage stocks infected far less cells than the same dilute passage stock alone. We can only conclude that undiluted passage vesicular stomatitis virus stocks contain a large proportion of a non-plaque-forming component which can interfere with virus growth or release. There is much less in dilute passage seeds, and it is self-duplicating or transmissible in the sense that it stimulates its own formation by infective virus and is not diluted out on successive passage but tends to increase in amount.

The analogy with the familiar ‘incomplete’ form of influenza virus seems very strong. However, because another type of interfering component exists (‘interferon’, Isaacs & Lindenmann, 1957), and because this interfering substance of vesicular stomatitis virus is not yet demonstrably ‘virus’ in serology and size, it will be called the transmissible interfering component, ‘T’. Some relations between T, incomplete influenza virus and interferon are discussed by Bellett & Cooper (1959).

The way in which T is made remains unknown. Ultraviolet, ‘storage’ (−20°) and thermal (37° and 50°) extracellular inactivation of infective vesicular stomatitis virus does not create T; nor does the inactivated virus produce much T when allowed to react with cells; the one passage at 41° (IND.UP. 34, Table 2) suggests that thermal intracellular inactivation gives similar results. Also the high yields on further undiluted passage of this stock and that stock made at 32° (IND.UP. 15) suggests that T synthesis is slower than infective virus synthesis at temperatures away from the optimum. T is made only slowly, if at all, in cells not containing infective virus, as otherwise one could not obtain T-free stocks by passage sufficiently diluted to make first-cycle mixed infection between T and infective virus unlikely, but insufficiently diluted to separate them entirely by end-dilution. This also suggests that T is not simply a non-cytopathogenic variant of the virus. Placing infective virus on all cells (thereby insuring at least some first-cycle mixed infection between T and infective virus), on the other hand, clearly allows T to be reproduced well. Only a minority of such cells liberates infective virus; which group liberates T remains unknown.

Regarding the way in which T reduces the yield of a culture, those cells yielding virus release a normal number of plaque-forming units although the release rate may be slower. Very many ‘infected’ cells cannot release infective virus at all, and exclusion in this sense accounts for nearly all the depression of yield. The widespread cytopathic effect shows that all cells were indeed ‘infected’, and that the infection, however aborted, is lethal. Exclusion is also shown by the protection of all cells in the three New Jersey low-dilution passages where less than 1 pfu was released per cell, and where cytopathic effect was absent. These indicate that T is not toxic by itself, and suggest that cell breakdown may not occur if enough time is allowed between adsorption of T and of infective virus.
Thus interference by T may take three forms: (a) a slowing of release of infective virus, (b) 'exclusion' (no release) with cell degeneration, (c) exclusion with no cell degeneration. These may conceivably reflect differences in time and quantities of adsorption of T and plaque-forming units, or different forms of T. The delayed degeneration in PI. 2, fig. 4 arose with a multiplicity of infective virus of 0.8–1, where for probably half the cells at least 4 hr. elapsed between receiving T and infective virus. Cytopathic effect was less delayed for diluted (10⁻³) T-free stocks, or for T-containing stocks of higher plaque-forming titre where a much shorter interval would elapse between adsorption of T and of infective virus.

It is interesting that ultraviolet, heat or 'storage' inactivated (and probably live also) dilute passage vesicular stomatitis virus cannot rapidly exclude homotypically (Cooper, 1958a), yet T can. Among other viruses, one wonders in how many cases of interference by killed or live homologous virus the presence of undetected T-like components can be ruled out.

Because of many genetically 'incomplete' bacterial strains, Burnet (1955) felt that all viruses may exist in some sort of incomplete form, but in its original meaning (von Magnus, 1951) of a transmissible non-cytotoxic interference it is harder to find without a haemagglutinin; reproducible growth and assay systems helped the recognition of T in vesicular stomatitis, in which it is not very obvious. Nevertheless, T-like components may occur among animal viruses other than myxoviruses. Mims (1956) concluded from results similar to the above that the transmissible interfering component of Rift Valley fever was 'incomplete' virus. However, environmental factors complicate in vivo interpretations, and the interfering component may not be the one reacting with or producing specific antibodies, so that as with vesicular stomatitis virus direct evidence of incomplete virus is lacking. Chambers (1957) showed that persistence of western equine encephalomyelitis virus in strain 'L' cells for many subcultures was due to a transmissible interfering component protecting most cells from cytopathic effect. Dilute inocula caused more cytopathic effect than less dilute, but thermally inactivated virus was not ruled out as the protective agent. On subculture in antiserum, and occasionally spontaneously, virus was lost and the cells became susceptible again, so that lysogeny or genotypic cell resistance seem excluded. Analogous persistence in a continuously growing culture might also be expected with vesicular stomatitis virus because (a) T can protect most cells from virus, (b) some cells receiving T and virus can yet release progeny virus, (c) T can be serially transmitted for many passages. T-like components could well account for instances of persistence in other viruses.

REFERENCES


P. D. Cooper & A. J. D. Bellott—Transmissible Interfering Component,
Plate 1
(Facing p. 496)
P. D. Cooper & A. J. D. Bellett—Transmissible interfering component.
Plate 2
Transmissible interfering component


EXPLANATION OF PLATES

Plates 1, 2, figs. 1–4 are phase-contrast pictures of living unstained chick embryo cell monolayers all of one routine batch, treated simultaneously in a manner identical except for type of inoculum. ×280.

**PLATE 1**

Fig. 1. Typical 20 hr. monolayer at usual stage of inoculation with virus. Cells are partially spread and nearly confluent.

Fig. 2. Typical 46 hr. monolayer, uninoculated. Cells are very well spread and confluent.

**PLATE 2**

Fig. 3. 46 hr. monolayer, 26 hr. after inoculation with 0.5 ml. IND. DP. 18 (2.7 × 10⁹ pfu/ml) and overlaying with 4.5 ml. medium. Cytopathic effect is maximal or very extensive in all cells, and many had resuspended from the glass. This effect is typical of most undiluted passage and all dilute passage stocks with inocula undiluted or diluted to 10⁻³.

Fig. 4. 46 hr. monolayer, 26 hr. after inoculation with 0.5 ml. IND.UP. 11 (5 × 10⁷ pfu/ml). Most cells showed some cytopathic effect being either rounded or with granular cytoplasm but without the extensive degeneration of Fig. 3. The culture had degenerated by 120 hr.

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Some Properties of the Transmissible Interfering Component of Vesicular Stomatitis Virus Preparations

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SUMMARY: In vesicular stomatitis virus inocula containing the transmissible interfering component (T) an exponential relation, at low doses, between inoculum concentration and virus yield suggested that adsorption of one T particle was enough to exclude infective virus. This relation was not maintained at high doses of inoculum, possibly because maximal interference required several hours between adsorption of T and virus particles. An assay method for T, based on the dose which gave 37% of the yield from T-free inocula, showed that the T content of inocula was usually related to passage history and yield of virus on undiluted passage. Treatment with immune serum did not appear to neutralize T, which sedimented in the centrifuge more slowly than did virus. T was much less rapidly inactivated at 56° and by u.v. irradiation than was the infectivity. Despite resemblances between the agent T, incomplete influenza virus and interferon, direct evidence is lacking that T is an incomplete form of the vesicular stomatitis virus or an interferon-like substance.

In a previous paper (Cooper & Bellett, 1959) we described a phenomenon with vesicular stomatitis virus which resembled the interference due to ‘incomplete’ virus in influenza (von Magnus, 1951). It was concluded that the low yields from serial undiluted passage of virus were due to a transmissible interfering component (T). The present paper attempts a further definition of the properties of the agent T, and a comparison of T with incomplete influenza virus and influenza interferon (Isaacs & Lindenmann, 1957).

METHODS

The preparation of media, virus stocks and tissue cultures, and the assay of virus by plaque count were described previously (Cooper, 1957; Cooper, 1958a; Cooper & Bellett, 1959). Virus titres are expressed as plaque-forming units (pfu)/ml., dilutions being made in phosphate-buffered saline (PBS).

Assay of the interfering agent T. Samples (0.5 ml.) of the virus stock to be assayed in twofold serial dilutions in the medium composed of Earle's saline + 5 mg. lactalbumin hydrolysate/ml. + 2 mg. yeast extract/ml. + 5% (v/v) horse serum were added to washed confluent (20 hr.) chick embryo cell monolayers. After 1 hr. at 37° 5 ml. of ‘conditioned’ medium (fluid from 20 hr. monolayers) were added, and 20 hr. later the supernatant fluids were removed and plaque-assayed immediately, or after 2–4 days at −20°. The derivation of the content of T agent from these values is discussed below.

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**Immune serum.** Standardized hyperimmune guinea-pig sera prepared with the Indiana or New Jersey serotypes of vesicular stomatitis virus were kindly supplied by Dr J. B. Brooksby (Research Institute, Pirbright, Surrey). These sera in a final dilution of 1/300 prevented plaque formation by homotypic virus, but not by heterotypic virus.

**Complement-fixation tests.** Complement-fixation tests (Brooksby, 1952) determined colorimetrically the haemolysis of sensitized sheep erythrocytes by complement left unixed by antigen+serum mixtures. A series of 1 ml. volumes of 1-25-fold dilutions of preserved guinea-pig serum (Wellcome Laboratories) in veronal buffer was incubated for 1 hr. with 0-2 ml. antigen and 0-4 ml. of 1/16 dilution of hyperimmune serum in colorimeter tubes in a water bath at 37°. Haemolytic serum (Wellcome Laboratories) diluted 1/100 was added to an equal volume of 3 % (v/v) sheep red blood cells and put in a water bath at 37° for 30 min. Two ml. of this sensitized red cell suspension were added with mixing to each colorimeter tube, and the tubes were left at 37° for a further 30 min. for haemolysis, then centrifuged at 2000 rev./min. for 5 min. and the colour of the supernatant immediately compared with distilled water in a Hilger-Spekker absorptiometer using a green filter. The points of 50 % haemolysis were obtained graphically using a probit plot and compared with a control complement titration to calculate the amount of complement fixed. Results (Table 6) are expressed in arbitrary units/ml., the units representing the difference between relative concentrations of complement required to give 50 % haemolysis in presence and absence of antigen (concentration in absence of antigen =1).

**High-speed centrifugation to separate T from infective virus.** Samples of undiluted passage virus stocks (3 ml.) were added to 5 ml. capacity glass centrifuge tubes which each contained 1 ml. of solidified agar gel (20 g./l. distilled water), and centrifuged at 13,500–14,000 rev./min. for 65 min. in a high-speed angle head ('International' refrigerated centrifuge) at 5°. The top 2 ml. of the supernatant was withdrawn for assay of T, plaque-forming units and complement-fixing activity.

**RESULTS**

The number of T particles required to exclude virus

Cooper & Bellett (1959) concluded that the low yields from serial undiluted passage of vesicular stomatitis virus stocks were caused by a transmissible interfering component, T. This will be considered to be particulate, even though it may be of molecular dimensions. Since it was also shown that the interference was mostly an all-or-none exclusion, where cells either released a normal yield or none at all, the ratio of the overnight yield from a T-containing inoculum to that from a T-free control can be taken as approximately the proportion of cells which did not receive an effective dose of T. The T-free control can either be a dilute passage stock, or the T-containing stock diluted sufficiently to make first-cycle mixed infection between T and plaque-forming units negligible.
A plot of the relative concentration of dilutions of T added to a series of monolayers (the stocks being serially diluted down to doses at which further dilution did not increase the yield) against the logarithm of yield relative to the maximum yield (multiplicity of infective virus being kept constant at 2.5) showed a negative exponential relationship between dose and cells remaining uninterfered with, suggesting that only one T particle per cell was required to exclude virus (Fig. 1). Experiments with some other T-containing stocks (together with one which was free of T), similar to that of Fig. 1 except that multiplicity of infection was not constant, showed that this exponential relation applied generally at low, but very rarely at high, doses (Fig. 2). This is discussed in the next section.

**Fig. 1.** The effect of T concentration on virus yield with a constant multiplicity of infective virus. Mixtures of NJ.UP.17 and NJ.DP.12 were prepared so that each mixture contained $10^8$ pfu/ml, while the T content varied from $3.2 \times 10^8$/ml to $2.7 \times 10^6$/ml. Inocula of 0.5 ml of each mixture were added to 20 hr. monolayers, 5 ml medium added after 1 hr. adsorption at 37°, and the supernatants plaque-assayed after a further 20 hr. at 37°. The highest multiplicity of T was 4 particles per cell, but the multiplicity of infective virus was always 2-5 pfu/cell. Relative yield is calculated on the basis of 2 x 10⁷ cells/monolayer, although not all cells yield virus.

**Fig. 2.** The effect of dilution of inoculum on yield for a number of virus stocks. T and infective virus were thus both varied together. The values of M refer to the multiplicity of infective virus with undiluted stock as inoculum (dose = 100). Δ = NJ.UP.17, ▲ = NJ.UP.16α, □ = NJ.DP.12, ■ = NJ.DP.8, ● = IND.UP.22, ○ = NJ.DP., stock, not numbered. Relative yield is calculated on the basis of 2 x 10⁷ cells/monolayer, although not all cells yield virus.

**The assay of T**

As mentioned above, the ratio between overnight yields from T-containing and T-free inocula can be taken as the proportion of cells which did not receive an effective dose of T, provided that enough time is allowed for virus release from all unprotected cells to be complete. When a culture has received an
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average of one effective dose of T/cell, the proportion of cells not receiving an effective dose of T, i.e. the proportion of yielders, should equal \( e^{-1}(=0.37) \). Since 1 effective dose = 1 particle (Figs. 1, 2), the inoculum dilution giving a 37% yield will contain about \( 4 \times 10^7 \) T particles/ml., since 0.5 ml. is always added as inoculum to monolayers containing about \( 2 \times 10^7 \) cells. From this the T content of the undiluted stock could be calculated; in those stocks with apparently low efficiencies of interference at high doses, the 37% yield was estimated from the low-dosage part of the curve. It must be recognized that (like plaque-forming units) the 'T particle content' is defined only by the assay system used, and, in common with any other biological activity estimated in terms of particles, is likely to represent a minimum due to probability effects and other causes of inefficient assay.

The following section shows that several such assays give results in agreement with expectations from undiluted passage yield and passage history of the stock. However, most assays involved simple serial dilution of the stock, so that low doses of T accompanied low doses of virus, and a later section suggests that an interval of several hours between receiving T and plaque-forming units is needed for maximal interference. The reduced effectiveness of T at high doses may be because a small variable proportion of cells is insusceptible to T but susceptible to virus, or because T is antagonized by some component of the stock, but it is more likely to be because the higher doses infect more cells in the first cycle (i.e. within say 30 min. of T). If T requires more time than this to protect all cells, then those cells infected in the first cycle will be more likely to succumb to virus. In support of this are the two assays of T in NJ.UP.17 afforded by Figs. 1 and 2. In Fig. 1 all cells received plaque-forming units within a short time of T, and the apparent T content was \( 1.5 \times 10^8 \) T particles/ml.; in Fig. 2, at the dose giving 37% yield probably less than 1% of the cells received plaque-forming units within 4–5 hr. of T, and the apparent T content was \( 6.4 \times 10^8 \) T particles/ml.

Presumably this perturbation will not affect the T assay if the curves are linear in that segment where the input multiplicity of live virus is appreciably less than 1. However, several curves are non-linear in this segment (Figs. 2, 3), so that, unless T is still not fully established by the third cycle, some other factor may also neutralize T. Therefore, while this assay does permit some interpretations in terms of absolute T particle concentrations, one can summarize its defects as (a) it requires a lengthy series of plaque assays and several assumptions, (b) it is interfered with by infective virus, (c) its reproducibility may be further affected by some other unknown factor.

Relation between T content, yield on undiluted passage and passage history

This relationship is summarized for a number of stocks in Table 1. As may be expected from the data of Cooper & Bellett (1959), stocks giving high yields on undiluted passage generally had high plaque-forming unit and low T contents, and stocks giving low yields had the reverse. Consequently, high-yielding stocks often had low T:plaque-forming unit ratios and vice versa.
Table 1 may suggest that this ratio is more closely related to undiluted passage yield than are absolute T or plaque-forming unit contents, but the data are here insufficient to show whether infective virus could compete with T and so neutralize the interference.

Table 1 also shows that serial undiluted passage usually resulted in a high, and dilute passage or a single undiluted passage a low, T content. The T content of dilute passage stocks depended on the dilution of passage, as may be expected, but some stocks passed at quite high dilutions (e.g. NJ.DP. 12) had a relatively high T content.

### Table 1. Comparison of history of several vesicular stomatitis virus stocks with T content and autointerfering ability

<table>
<thead>
<tr>
<th>Stock</th>
<th>Yield on undiluted passage (pfu/cell)</th>
<th>T content (particles/ml)</th>
<th>Infective titre (pfu/ml)</th>
<th>T:pfu ratio</th>
<th>History</th>
</tr>
</thead>
<tbody>
<tr>
<td>IND.UP.22</td>
<td>500</td>
<td>4.8 x 10^7</td>
<td>2 x 10^9</td>
<td>1:42</td>
<td>1 UP from one pfu</td>
</tr>
<tr>
<td>NJ.DP.14</td>
<td>375</td>
<td>&lt; 10^2</td>
<td>2.4 x 10^9</td>
<td>1: &gt; 260</td>
<td>1 DP from one pfu</td>
</tr>
<tr>
<td>IND.DP.3</td>
<td>60, 72, 205</td>
<td>c.10^8</td>
<td>3.2 x 10^9</td>
<td>1:3-2</td>
<td>1 UP + 1 DP (1/3) from one pfu</td>
</tr>
<tr>
<td>NJ.DP.8</td>
<td>10, 250</td>
<td>3.1 x 10^9</td>
<td>2.7 x 10^9</td>
<td>1:8-8</td>
<td>1 DP (1/2) from original stock</td>
</tr>
<tr>
<td>IND.UP.13</td>
<td>70</td>
<td>5.6 x 10^8</td>
<td>2.4 x 10^9</td>
<td>2:3:1</td>
<td>Long UP + very low-dilution passage series from 1 pfu</td>
</tr>
<tr>
<td>NJ.UP.17</td>
<td>55</td>
<td>1.5 x 10^8</td>
<td>1 x 10^9</td>
<td>1:5:1</td>
<td>Artificial mixture of NJ.UP. 17 plus</td>
</tr>
<tr>
<td>NJ.DP.12</td>
<td>22</td>
<td>2.5 x 10^8</td>
<td>2.1 x 10^9</td>
<td>1:8-4</td>
<td>1 UP + 3 very high-dilution passages from 1 pfu</td>
</tr>
<tr>
<td>IND.DP.24</td>
<td>6</td>
<td>2.8 x 10^8</td>
<td>1 x 10^9</td>
<td>1:3-6</td>
<td>Long high-dilution passage series</td>
</tr>
<tr>
<td>NJ.DP.12a</td>
<td>2, 2</td>
<td>4 x 10^9, 8 x 10^9</td>
<td>1.9 x 10^6</td>
<td>210:1</td>
<td>Long UP. or low-dilution passage series from 1 pfu</td>
</tr>
<tr>
<td>NJ.UP.17</td>
<td>1·6</td>
<td>6.4 x 10^9</td>
<td>7·2 x 10^9</td>
<td>89:1</td>
<td>Long UP. or low-dilution passage series from 1 pfu</td>
</tr>
</tbody>
</table>

*Time required for establishing exclusion by T*

Although this time was not measured directly, some indications of its duration have been obtained. In one series of monolayers (Table 2) inoculated simultaneously with a constant amount of T and varying amounts of infective virus, those cultures with an average input multiplicity of infection considerably less than one gave a yield appreciably less than those with multiplicities around one, despite greater chances for thermal inactivation in the latter.
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Thus, although interference was present in all cultures, those cells which were not infected until 4–6 hr. after receiving T (i.e., until the second cycle) were more resistant to the virus. The culture with the lowest inoculum showed some delay in cytopathic effect, a phenomenon never found with comparable inocula of dilute passage virus.

Table 2. Effect on yield of varying the multiplicity of infective virus,
T being kept constant

A series of monolayers was infected with 0.5 ml. each of mixtures containing constant amounts of T but different amounts of plaque-forming units. After 45 min., 5 ml. of medium were added, and the supernatants harvested for plaque assay after a further 20 hr.

<table>
<thead>
<tr>
<th>Inoculum per cell</th>
<th>Yield (pfu/cell)</th>
<th>Cytopathic effect at 20 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfu T particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2 4.9</td>
<td>18</td>
<td>++</td>
</tr>
<tr>
<td>1.6 4.5</td>
<td>18</td>
<td>++</td>
</tr>
<tr>
<td>0.85 4.3</td>
<td>42</td>
<td>+ +</td>
</tr>
<tr>
<td>0.44 4.1</td>
<td>42</td>
<td>+ +</td>
</tr>
<tr>
<td>0.24 4.1</td>
<td>5.8</td>
<td>+ +</td>
</tr>
<tr>
<td>0.14 4.0</td>
<td>9.0</td>
<td>+ +</td>
</tr>
<tr>
<td>0.045 4.4</td>
<td>2.0</td>
<td>±</td>
</tr>
</tbody>
</table>

This protection was also found several times with undiluted passage virus where most cells were not infected in the first cycle (Cooper & Bellett, 1959). In experiments where T and plaque-forming units were added simultaneously to all cells, about 80% of cells were prevented from releasing virus, but not protected from cytopathic effect. We therefore conclude that the bulk of the exclusion is established within 1 hr., but that longer, perhaps 6–8 hr., is required to protect cells fully (especially from cytopathic effect). These times may well depend on relative multiplicities of T and plaque-forming units; interference is the resultant of the two processes of establishing ‘infection’ by T and by infective virus, and as mentioned by Cooper (1958b), one cannot deduce much about the rates of the two component processes from the apparent rate of establishing interference.

Heterotypic interference by undiluted passage virus stocks

Three groups of monolayers received either (a) Indiana virus alone, or (b) Indiana virus plus a little New Jersey virus plus New Jersey T particles in excess, or (c) Indiana virus plus the same small addition of New Jersey virus as an extra control (Table 3). All monolayers had the same multiplicity of Indiana virus. As the infective New Jersey virus was present in minor amounts and is less effective than Indiana virus in establishing exclusion (Cooper, 1958b), its contribution to yield and infective centres was ignored; immune serum was not used. Table 3 shows that the presence of undiluted passage New Jersey virus considerably depressed both yield and infective centres formed by Indiana virus, in amounts not due to the small presence of New Jersey infective virus. The interference was only partly accounted for by the
decrease in the number of infective centres established by the dilute passage Indiana virus. Thus, undiluted passage New Jersey virus has a heterotypic interfering activity not shown by dilute passage New Jersey virus at the same multiplicity, although the relationship between the heterotypic excluding agent and T remains to be demonstrated.

Table 3. Heterotypic interference by undiluted passage virus stocks

<table>
<thead>
<tr>
<th>Group</th>
<th>Inocula</th>
<th>Infective centres/monolayer $\times 10^6$</th>
<th>Total cells/monolayer $\times 10^7$</th>
<th>Mean* plating efficiency</th>
<th>Total yield (pfu/monolayer) $\times 10^4$</th>
<th>Mean† yield (pfu/infected cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IND virus (M = 0.5)</td>
<td>5.9</td>
<td>1.6</td>
<td>8.2</td>
<td>120</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>IND virus (M = 0.5) + NJ virus (M = 0.09) + NJ T particles (M = 8)</td>
<td>1.9</td>
<td>1.8</td>
<td>3.4</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>IND virus (M = 0.5)</td>
<td>6.5</td>
<td>1.4</td>
<td>8.2</td>
<td>120</td>
<td>1580</td>
</tr>
<tr>
<td></td>
<td>IND virus (M = 0.5) + NJ virus (M = 0.09) + NJ T particles (M = 8)</td>
<td>3.0</td>
<td>1.5</td>
<td>3.4</td>
<td>15</td>
<td>280</td>
</tr>
<tr>
<td>3</td>
<td>IND virus (M = 0.5)</td>
<td>7.5</td>
<td>2.8</td>
<td>9.8</td>
<td>120</td>
<td>860</td>
</tr>
<tr>
<td></td>
<td>IND virus (M = 0.5) + NJ virus (M = 0.11)</td>
<td>9.4</td>
<td>2.8</td>
<td>9.8</td>
<td>30</td>
<td>860</td>
</tr>
</tbody>
</table>

* Plating efficiency = ratio of infective centres expected from inoculum: infective centres found.
† Based on infective centre count corrected for plating efficiency in control (Group 1), i.e. col. 3 is multiplied by 100/8.2.

The failure of immune serum to neutralize heterotypic interference by undiluted passage virus stocks

Three confluent monolayers received either (a) Indiana virus + Indiana T particles, (b) Indiana virus + Indiana T particles + Indiana antiserum, or (c) medium alone, as primary inocula (Table 4). After adsorption they were challenged with New Jersey virus as secondary inocula, and the supernatant fluids assayed for New Jersey plaque-forming units after virus growth and release. Table 4 shows that the Indiana antiserum did not prevent the Indiana undiluted passage virus from interfering with growth of New Jersey virus.
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Table 4. The failure of immune serum to neutralize heterotypic interference by undiluted passage virus stocks

One 20 hr. monolayer received 0·5 ml. IND.UP. 49 (1·7 × 10⁸ pfu/ml., 4·4 × 10⁸ T/ml.), another received 0·5 ml. IND.UP. 49 plus 0·2 ml. Indiana antiserum (1/2 PBS), and a control monolayer received 0·5 ml. ES. After 30 min. at 37°, 0·5 ml. NJ.DP. 14 diluted 1/10 ES (2·6 × 10⁸ pfu/ml.) was added to each monolayer as secondary inoculum and 5 ml. medium were added 30 min. later. The fluids were harvested and titrated 8 hr. after infection, Indiana antiserum (0·2 ml. of 1/10 dilution in PBS) being added to the agar overlayers.

<table>
<thead>
<tr>
<th>Primary inoculum</th>
<th>Secondary inoculum</th>
<th>Yield (pfu/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0·5 ml. IND.UP. 49</td>
<td>0·5 ml. NJ.DP. 14 (10⁻¹)</td>
<td>5·2 × 10⁷</td>
</tr>
<tr>
<td>0·2 ml. IND. antiserum (1/2)</td>
<td>0·5 ml. NJ.DP. 14 (10⁻¹)</td>
<td>3·4 × 10⁴</td>
</tr>
<tr>
<td>0·5 ml. ES</td>
<td>0·5 ml. NJ.DP. 14 (10⁻¹)</td>
<td>2·5 × 10⁶</td>
</tr>
</tbody>
</table>

The effect of heat and ultraviolet treatment on homotypic interference by undiluted passage virus stocks

Vesicular stomatitis virus minimally inactivated by heat or ultraviolet light, and therefore probably live virus also, does not interfere with the multiplication of infective virus of the same serotype if added simultaneously (Cooper, 1958b). If an undiluted passage stock is treated with heat or u.v. it should therefore be possible to follow the effect of the treatment on the interfering ability of T without complication from interference by inactivated virus.

Table 5 describes an experiment in which a T-containing New Jersey stock was exposed to u.v. or heated at 56° for varying times. After treatment samples

Table 5. Heat and ultraviolet treatment of undiluted passage virus stocks

A stock of NJ.UP.16 virus (9·0 × 10⁷ pfu/ml.) was divided into 1 ml. samples in 5 ml. screw-capped bottles. One bottle was removed to 4° in the dark as a control; some bottles were put in a 50° water bath and others, with their caps removed, were put 50 cm. below a Philips 30 W. u.v. lamp. All were frequently shaken. After the exposure times shown, samples were removed to 4° in the dark and then titrated by plaque count. Half (0·5 ml.) of each sample was added to a monolayer, with medium alone on another as control, and all were followed 10 min. later by 0·25 ml. NJ.DP.14 diluted 1/2 in medium (giving 16 pfu/cell), and 5 ml. medium after a further 30 min. at 37°. The yields were harvested and titrated 20 hr. later.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample time (min.)</th>
<th>Residual virus (pfu/ml.)</th>
<th>Yield from 0·5 ml. samples + 0·25 ml. NJ.DP.14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>9·1 × 10⁷</td>
<td>1·3 × 10⁹</td>
</tr>
<tr>
<td>56°</td>
<td>5</td>
<td>8·8 × 10⁴</td>
<td>1·6 × 10⁸</td>
</tr>
<tr>
<td>56°</td>
<td>10</td>
<td>1·2 × 10⁴</td>
<td>1·3 × 10⁹</td>
</tr>
<tr>
<td>56°</td>
<td>15</td>
<td>2·1 × 10⁴</td>
<td>1·8 × 10⁸</td>
</tr>
<tr>
<td>56°</td>
<td>20</td>
<td>1·6 × 10⁴</td>
<td>2·3 × 10⁸</td>
</tr>
<tr>
<td>56°</td>
<td>25</td>
<td>1·0 × 10⁴</td>
<td>1·6 × 10⁸</td>
</tr>
<tr>
<td>u.v.</td>
<td>10</td>
<td>2·7 × 10⁵</td>
<td>4·0 × 10⁷</td>
</tr>
<tr>
<td>u.v.</td>
<td>20</td>
<td>7·3 × 10⁷</td>
<td>2·4 × 10⁷</td>
</tr>
<tr>
<td>u.v.</td>
<td>30</td>
<td>2·0 × 10⁹</td>
<td>3·0 × 10⁸</td>
</tr>
</tbody>
</table>
were at once compared with an untreated control for plaque-forming unit contents, and the infectivity was found to decrease approximately exponentially with time of treatment in both cases. The full series of assays required for quantitative T comparisons were not performed, but the interfering abilities were compared by their effect on the overnight yield from a standard New Jersey inoculum. Table 5 shows that no detectable effect on interference followed a u.v. dose decreasing the plaque-forming unit content to 2% of the original. On the other hand, a large drop in infectivity at 56° (to $10^{-4}$ of the original) accompanied a progressive but not complete drop in interfering activity. We conclude that T is much more stable to u.v. and 56° inactivation than is the infectivity, but at doses equally lethal for infectivity a temperature of 56° is probably a more effective inactivator of T than is u.v.

**Separation of T and plaque-forming units by centrifugation**

Samples of clarified medium and disrupted cells from monolayers infected with a T-containing inoculum, harvested early (8 hr. after infection) to minimize contamination with 'soluble antigen', were centrifuged as described so as to sediment most but not all of the infectivity. The products were compared with uncentrifuged samples with respect to infectivity, T content and complement fixation (Table 6 and Fig. 3). It was hoped thus to separate T from plaque-forming units, and to indicate a method for separating T from the bulk of the complement-fixing activity in order to study the serological nature of T.

The cell debris had a high complement-fixing activity which was not deposited on centrifuging, and had a low plaque-forming activity 90% of which

---

**Table 6. High speed centrifugation of an 8 hr. undiluted passage virus stock**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Titre complement (pfu/ml.)</th>
<th>Units complement fixed/ml.</th>
<th>T particles/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium before centrifugation</td>
<td>$1.9 \times 10^4$</td>
<td>16-0</td>
<td>$8.0 \times 10^3$</td>
</tr>
<tr>
<td>Medium, supernatant from 65 min. at c. 14,000 rev./min.</td>
<td>$2.0 \times 10^4$</td>
<td>12-3</td>
<td>$6.6 \times 10^3$</td>
</tr>
<tr>
<td>Cell debris before centrifugation</td>
<td>$1.0 \times 10^6$</td>
<td>76-2</td>
<td>Not assayed</td>
</tr>
<tr>
<td>Cell debris supernatant from 65 min. at c. 14,000 rev./min.</td>
<td>$1.4 \times 10^4$</td>
<td>83-0</td>
<td>Not assayed</td>
</tr>
</tbody>
</table>
Transmissible interfering component

was deposited. From the medium containing released virus c. 90% of the infectivity was also deposited, while the complement-fixing activity of the supernatant dropped by about 20%. The T content of the tissue culture fluid was also decreased by 20% on centrifuging.

It can be concluded that the interfering activity characteristic of T was present in the 8 hr. yield of undiluted passage virus and had a much smaller sedimentation constant than the infective virus. Little quantitative significance can be attached to the differences in complement fixing activity or T content before and after centrifuging the preparations; they indicate that T is not readily separated from virus antigen by centrifugation, precluding for the present any identification of T with virus serological activity because of probable contamination with soluble antigen (Bradish, Brooksby & Dillon, 1956).

Fig. 3. Relation between yield and inoculum dilution for a T-containing stock before (○) and after (•) removal of 90% of the plaque-forming units by centrifugation (for details see Table 6). Serial dilutions of the two samples were added to monolayers in 0.5 ml. amounts, and 5 ml. medium added after 30 min. adsorption. Medium was harvested for assay after 22 hr. Relative yield is calculated on the basis of 2 x 10⁷ cells/monolayer, although not all cells yield virus.

DISCUSSION

These results provide a little more information about the way in which the agent T excludes infective virus, and about the properties of the T particle itself. The exponential relation between dose of T and yield of virus at low doses indicates that only one T particle needs to be adsorbed to a cell for infective virus to be excluded. This high efficiency is quite different from the heterotypic interference of dilute passage vesicular stomatitis virus (Cooper, 1958b) where, although exclusion occurs, interfering and challenge virus appear to compete, and adsorption of many interfering particles is needed to exclude a single challenge particle; this may reflect differing rates of
establishing exclusion and infection. However, at high doses of undiluted passage virus T does not protect as many cells as would be expected, perhaps because more cells receive infective virus before T has had time to establish itself. Nevertheless, even when all cells have received infective virus at an early time (Fig. 1) the relationship is still linear; thus although fewer cells have received T in time so that fewer cells are protected, the protection is still efficient in the sense that only one particle appears to be adequate.

The presence of so much T component in the medium 8 hr. after infection (Table 6) means that the production and release of T must be broadly coincident with that of infective virus.

The homotypic interfering ability of T differs from the infectivity of intact virus in its much greater stability to heat and u.v. inactivation, but resembles the heterotypic interfering ability of intact virus in this respect. It is interesting that, in heterotypic tests, T was not neutralized by antiserum. T is also much less easily sedimented in the centrifuge than is virus, indicating that it is smaller, or less dense, or both. Its consequent contamination with soluble antigen has impeded serological identification as 'virus' by this means.

The formation of T by undiluted passage (Cooper & Bellett, 1959) suggested an analogy with incomplete influenza virus, and several other properties of T support this idea. However, most of the properties found are also shared with influenza interferon (Isaacs & Lindenmann, 1957); for example, all three have lower sedimentation constants than infective virus (Gard, von Magnus, Svedmir & Birch-Andersen, 1952; Isaacs, Lindenmann & Valentine, 1957) and all are relatively insensitive to heat or u.v. inactivation (Paucker & Henle, 1958; Burke & Isaacs, 1958; Isaacs, Lindenmann & Valentine, 1957). Interferon might be expected to be found after undiluted passage of influenza virus because of the presence of more heat-inactivated virus than in dilute passage; heat-inactivated virus stimulates formation of interferon (Isaacs & Lindenmann, 1957). None of the three forms is transmissible by itself, but interferon, like T and incomplete influenza virus, should be transmissible in indefinite subculture provided that it is passed with appropriate amounts of infective virus. Neither T nor interferon (Isaacs, Lindenmann & Valentine, 1957) is sensitive to immune serum; precisely comparable data seem to be unavailable for interference by incomplete influenza virus, although one might expect it to differ in this respect. Interferon (Isaacs, 1959) and T are not visibly cytotoxic by themselves; incomplete influenza virus is also probably nontoxic (von Magnus, 1954). Although incomplete influenza virus requires an 18–24 hr. interval before challenge to establish maximum interference, appreciable interference occurred after 1 hr. (von Magnus, 1954); interferon requires several hr. incubation at 37° for its effect to be maximal (Isaacs, 1959). T probably establishes most of its interference within 1 hr. although there was evidence that interference improved for a few hours after. It is difficult to compare the rates of development of interference in such different systems, but it seems that T resembles interferon more than incomplete influenza virus in this respect.

Interference in influenza virus is undoubtedly a complex thing, and may
Transmissible interfering component

involve even more components than those already discovered. At present there is unfortunately not enough evidence to conclude much about T by analogy with influenza virus; T could be an incomplete form, or an interferon, or some other entity quite different from these, although where comparison is possible there is more resemblance with interferon, particularly in its slow rate of sedimentation in the centrifuge.

REFERENCES


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The Bulk Growth of Animal Cells in Continuous Suspension Culture

BY P. D. COOPER, J. N. WILSON AND AVRIL M. BURT
SUMMARY: Several methods for growing animal cells in suspension culture were examined, to find the most efficient in terms of cells produced in a given time for the minimum of medium and attention. Continuous medium flow was more efficient than batch-culture, and the preferred system was to add medium to a culture vessel regularly in small doses via a time-switch-controlled solenoid closure at a rate similar to the growth rate; a turbidimetric safety device ensured that cell density did not drop below levels permitting growth. A mixture of galactose or fructose (6 g./l.) and glucose (2 g./l.) allowed better pH control than glucose alone (6 g./l.). Doubling times of 14–16 hr. were obtained on occasion; the gas-phase oxygen concentration for fastest growth depended on cell density and was frequently less than atmospheric, suggesting that these cells can behave as microaerophils.

Microbiologists depend upon an adequate supply of a growth medium for their microbes, and intact animal cells seem likely to remain essential for in vitro growth of animal viruses for some time. Unfortunately, cell supply often limits the work accomplished, so that a simple and cheap laboratory-scale method for bulk growth of continuous cell lines has some attractions. A similar case can be made for cells required for virus vaccine manufacture.

This paper describes an experimental approach to such a method, based on a continuous flow of medium to a culture of suspended cells. Emphasis has been placed on economy of operation, both in materials and attention, and the method should be capable of expansion to pilot-plant scale or contraction to the needs of a small laboratory; its feasibility has previously been reported briefly (Cooper, Burt & Wilson, 1958). Some aspects of the growth conditions of the cells are reported, particularly their very rapid growth under optimal conditions with a doubling time of less than 16 hr., and the inhibitory effect of oxygen excess which could occur at oxygen concentrations less than that in normal air. The use of these cells for poliovirus bulk growth and assay will be described separately.

METHODS

General scheme. The object was to overcome the bottle-neck of cell production in stationary cultures as cheaply and simply as possible, and continuous suspension culture seemed the best method. However, some ancillary bottle culture was still needed, and a simplified procedure for this is described. Four methods for suspension culture were tried: batch culture, continuous medium flow ("chemostat"), photo-electrically controlled discontinuous medium flow ("turbidostat"), and a combination of the last two ("chemo-turbidostat"). Their merits are compared below. The apparatus used for the turbidostat
Animal cells in continuous suspension culture

and the chemostat were similar, and the differences involved are described where appropriate. As emphasized below, reliability was essential; all the components described have proved reliable in almost continuous operation at 37° for between 9 and 25 months.

The apparatus is shown in Fig. 1; where possible, standard commercial products were used throughout. Pyrex aspirators (20 l.) acted as medium reservoirs (a), culture vessel (b) and receiver (c); b and c were stirred and gassed continuously. Aspirators b and c were silicone-treated before autoclaving; this lessened but did not prevent the accumulation of some cells at the culture meniscus. Medium was allowed to fall at intervals via a solenoid-operated spring closure (d) (Callow & Pirt, 1956) controlled by a time-switch ("chemostat") or by a photo-electric device ("turbidostat"). These are described in Figs. 2 and 3. Alternatively, medium was continuously pumped (chemostat) at a rate monitored by a rotameter (e) from a to b, which overflowed via the constant-level vent into c.

The inset within the dotted line shows alternative flow control to the solenoid and includes the pump (f), which consisted of a rotating ring of rollers pressing the rubber tubing against a pivoted plate kept at a constant pressure by a spring. The pumping rate was governed by the speed of rotation, dimensions of the tubing, spacing between the rollers, tension of the spring and the head of medium, the latter necessitating a Marriotte tube in the reservoir to keep the hydrostatic pressure constant. Wear by the rollers on the tubing was considerable. The Marriotte tubes were not necessary for the turbidostat. A rotameter calibrated in 5–150 ml./min. for air was adequate for 0.2–5 l. medium/24 hr. Cells grew in b in a shallow layer of medium of constant depth (5–7 cm.), but the depth in c increased as cells plus medium entered. The overflow from b must be removed rapidly, and therefore intermittently, rather than by a continuous siphon, as otherwise cells settled in the pipe. Harvesting (P) and replenishing ports (P') were dust-caps made of 37 x 55 mm. or 60 x 80 mm. aluminium screw-cap containers (Metal Box Co., London, W. 1) with stainless steel tubes bolted through, and could be thoroughly flamed when open or closed. Where possible all tubing and T-pieces, particularly those passing through bungs, were of stainless steel because of occasional accidental breakage of glass tubes. Outflows from culture vessel and receiver were ½ in. internal diam., other tubing was ¼ in. internal diam. All pipelines in contact with medium were of silicone rubber tubing (Esco Rubber Co., Ltd., London, N. 16), and air-lines were of ‘drab’ white rubber tubing (J. G. Ingram and Co., Ltd., London, E. 9); closure was by screw clips (g) on to tubing protected by cotton-backed adhesive tape. Sampling (X) and inoculating (Y) ports employed ½ oz. bottles (United Glass Bottles Ltd., Leicester Street, London, W.C. 2); the central threaded holder was a Hemmings filter (Hemmings Ltd., Beaumaris, Anglesey) with the centre perforated plate drilled out, and the top portion was an inverted ½ oz. bottle with the bottom removed and a bung inserted. The magnetic stirrer motors (Baird and Tatlock Ltd., Chadwell Heath, Essex) had the cover-plates discarded and speed-adjusting rheostats removed for remote operation to avoid local heating; the stirrers were barrel-shaped, weakly
Fig. 1. Assembly of laboratory apparatus used for continuous culture of ERK cells. The lines carrying air or a gas mixture are stippled; a, medium reservoirs; b, culture vessel; c, receiver; d, solenoid valve closure; e, rotameter; f, pump; g, screw clips; h, cotton wool plugs; i, anti-splash medium inlet; j, cooling jacket of rubber tubing; k, thermometers; A, gas inlet; B, gas outlet; D, light source; E, photocell; P, harvesting and P' replenishing ports. X and Y are sampling and inoculating ports respectively, shown schematically in the main diagram and in detail in the inset; samples were withdrawn via X or added via Y by sucking or blowing through the cotton wool filters. The bar across the medium line at S indicates the position of the sterile connection made during assembly.
Animal cells in continuous suspension culture

magnetic stainless steel (Esco Rubber Co., London, N. 16) or strongly magnetic iron covered with polytetrafluoroethylene (X-lon Products Ltd., London, S.W. 1). The latter were preferred, but they spin on a central tyre which wore flat in 3 weeks of continuous running; therefore the tyres were machined off and replaced with stainless steel rings. Apart from the sampling, harvesting, replenishing and inoculating ports, all openings to the air were protected by 2 x 15 cm. cotton-wool plugged tubes (h). To avoid surface bubbles which affected the photoelectric control, the medium inlet had an anti-splash device (i); it was preferred to keep the inlet visible to detect possible leaks through the solenoid closure. The gas outlet (B) was taken to the highest level possible to avoid spillage in case of undue leakage through the solenoid closure. The culture vessel (b) was covered with light-proof black cloth with lift-up flaps for inspecting stirring, volume and temperature. Thermometers (k) enclosed in b and c were tied to the sampling tubes with stainless steel wire so as to be away from the stirrer and more easily visible. The whole device was mounted on a frame in a room at 35°-36° with efficient air circulation; care was necessary to keep heat-producing electrical components away from the culture vessel, in which the temperature was 36°-37°. The receiver was cooled to between 28° and 32° by water pumped through a jacket of rubber tubing (j) from a water bath (Grant Instruments Ltd., Cambridge), which was cooled by copper coils through which mains water passed and was heated thermostatically to about 27°. A small inverted aspirator filled with water acted as a constant-level device for the water bath, to replace evaporation losses.

Stirring. Continuous stirring was essential; relatively slow rates were adequate to suspend the cells, but to promote gas exchange a shallow layer with as rapid stirring as possible without frothing was preferred. The culture vessel stirring rate was kept constant at 250–300 rev./min., using a 6 cm. stirrer, but the receiver stirring rate was increased as the depth of fluid increased. Extensive cell trauma was not found, and grinding was minimized by using a barrel-shaped or centre-tyred stirrer on the convex aspirator bottom; culture volumes of 1–2 l. rather than 100–200 ml. also minimized grinding, loss of cells on meniscus and difficulties of obtaining very low pumping rates. Even with such a shallow layer there was evidence that CO₂ removal was not adequate; this could probably be met by higher gas flow rates.

Aeration. A mixture of O₂, N₂ and CO₂ was obtained from cylinders with ‘BOR 12’ (British Oxygen Gases, Greenwich) regulators, which were sufficiently sensitive to control flow rate using a mercury manometer with fine capillary by-pass. The mixture, at a total flow of 100 ml./min. and monitored by a rotameter as a check on the manometers, was fed without deliberate moistening via a cotton-wool plug (Fig. 1, A) to 3 cm. above the culture surface; the oxygen demand of the culture would probably be met by 10 ml./min. Bubbling gas through the culture in the presence of antifoam agents was avoided because of risk to cell viability; varying the gas mixture could probably meet most oxygen needs.

Medium flow control. Flow in the chemostat was controlled by varying the speed of the pump or altering the duration or frequency of energizing the
solenoid by the time switch; the difficulties in judging the culture's needs are discussed below. The flow in the turbidostat depended on changes in the light reflected by the culture which increased with increase in cells; an increase in reflected light decreased the resistance of the photocell beneath the culture vessel, and the relay operating the solenoid was actuated when the resistance fell below a pre-set value. The optical arrangement is shown in Figs. 1 and 2; two ORP. 90 cadmium sulphide photocells (Mullards Ltd., London, W.C. 1) were used to minimize effects of variation in light source intensity, resulting particularly from mains voltage variation, which had an appreciable effect on the light even with a constant-voltage transformer (Advance Components Ltd., Ilford, Essex). The light source was an intense light microscope lamp (C. Baker and Co., Croydon, Surrey) with a 6 V. 50 W. bulb with five filaments,

![Diagram](image)

Fig. 2. Plan of optical arrangement for turbidimetric control; the large circle represents the culture vessel and the central bar the stirrer. Light from the bulb (a) passes through heat-resistant (b) and red (c) filters and a focusing lens. Some light is reflected by a sheet of plain glass (d) on to the balancing photocell (e), and the remainder passes into the culture over the controlling photocell (f).

so that failure of one filament would not inactivate the control, and was run at 75% of rated voltage to increase filament life. As shown in Fig. 2, the light beam entered a light-proof box through a heat-resisting (b) and a red (c) filter (no. OR/2, Pilkington Glass Co., London, S.W. 1) and was partly reflected by a plain glass plate (d) on to the balancing photocell (e); most of the light entered the culture from the side and was reflected by the tissue cells on to the photocell beneath (f). Two alternative electrical control systems were used; one (Fig. 3a) embodied a Multilec controller-recorder (George Kent Ltd., Luton, Beds.) with a knob which could control cell density, coupled to the output of a stable pH meter (Model 23 A, Electronic Instruments Ltd., Richmond, Surrey); the other (Fig. 3b) used a transistor relay (no. 596 HS (C)/TS. 5205, Magnetic Devices Ltd., Newmarket, Suffolk) across a Wheatstone's bridge, the cell density being varied by the variable resistances. Either system operated the solenoid closure on the medium line via a Sunvic (Sunbury, Middx.) relay no. F. 102/8 M. The first was used to establish the conditions for control from a continuous record, the second was a cheaper alternative suitable for duplication. A third and simpler but less stable circuit (Fig. 3c) was used to control turbidity in the 'chemo-turbidostat' using additionally a time switch (type MZM, Venner Ltd., New Malden, Surrey). An intermittent solenoid closure was
Animal cells in continuous suspension culture

preferred to a peristaltic pump, as there was much less wear on the tubing and it was easier to buy or build. A forced flow (e.g. by a pump) rather than continuous slow gravity flow was essential, as with such very slow flow rates gas bubbles constantly blocked the medium lines; no such trouble occurred with the rapid intermittent flows from the solenoid valve.

Medium. Advantage was taken of the considerable literature on media for cell culture (e.g. Morgan, 1958). However, most of such work has justifiably aimed at simplification, tending to 'minimal' media, but we have aimed at a 'maximal' medium (medium CSV.6, Table 1) giving fastest growth and highest
cell densities as cheaply as possible. Earle's saline was used as a basis (Earle et al. 1943) in which the salt concentrations are very similar to those found optimal by Eagle (1956). Lactalbumen hydrolysate (0-5 mg./ml.) was roughly equivalent to a 10- to 20-fold Eagle’s (1955) amino acid supplement if extra glutamine or glutamic acid plus NH₄Cl and methionine were added; extra arginine (Thomas, Ziegler, Schepartz & McLimans, 1958) and inositol were also included together with extra glucose and bicarbonate. The freezing-point of the medium was —0-55°. Most components were added to their highest non-toxic levels; the contents of K⁺ and PO₄³— should theoretically limit yield to about 2×10⁷ cells/ml., but this level has not been reached (mainly due to difficulties with pH control and glucose supply); and so no simplification has yet been attempted. Unlike the experience of Ziegler, Davis, Thomas & McLimans (1958), bicarbonate could not be omitted because of the high acid production by our cells; the other substances with appreciable buffering activity, lactalbumen hydrolysate and phosphate, were at their maximum non-toxic concentration. Although high cell density and ease of operation were the major aims, the growth rates achieved when gas mixtures were optimal were sometimes very rapid. All water was distilled in one step from hard mains water by a glass still (Loughborough Glass Co., Loughborough, Leics.). Horse serum, obtained from selected horses by venepuncture, and calf serum from a local abattoir, were Seitz-filtered without heat-inactivation before being added to the medium, which was then filtered through EK or EKS grade pads (Carlson Ltd., London). With this treatment no pleuropneumonia-like organisms were found in our cultures. Since medium often stood at 37° for several weeks, the penicillin level is high in CSV. 6; nystatin (Mycostatin, Squibb and Co., Ltd., Speke, Liverpool) was occasionally added sterilely to 25 μg./ml. after filtration but was not added routinely to the reservoirs because of possible accumulation of toxic oxidation products (Paul, 1959); otherwise all mixing was done before filtration.

Table 1. The components of medium CSV.6

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5-48</td>
<td>Biotin 0-001</td>
</tr>
<tr>
<td>KCl</td>
<td>0-5</td>
<td>Choline chloride 0-001</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0-2</td>
<td>Folic acid 0-001</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>0-2</td>
<td>Nicotinamide 0-001</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0-05</td>
<td>Calcium pantothenate 0-001</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2-5</td>
<td>Pyridoxal 5' phosphate 0-001</td>
</tr>
<tr>
<td>NaH₂PO₄.2H₂O</td>
<td>0-5</td>
<td>Thiamine 0-001</td>
</tr>
<tr>
<td>Difco yeast extract</td>
<td>1-0</td>
<td>Riboflavin 0-0001</td>
</tr>
<tr>
<td>Lactalbumen hydrolysate</td>
<td>5-0</td>
<td>Penicillin 375,000 units/l.</td>
</tr>
<tr>
<td>Glucose</td>
<td>6-0</td>
<td>Streptomycin 0-125</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0-1</td>
<td>Neomycin 0-10</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0-3</td>
<td>Phenol red 0-01</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0-1</td>
<td>Water To 1000 ml.</td>
</tr>
<tr>
<td>L-Arginine hydrochloride</td>
<td>0-05</td>
<td>Horse serum 75 ml.</td>
</tr>
<tr>
<td>Inositol</td>
<td>0-01</td>
<td>Calf serum 25 ml.</td>
</tr>
</tbody>
</table>

The sera were added after making up to 1 l.
Ancillary bottle culture. This has been retained to restart a culture machine with minimum delay in event of breakdown; otherwise cells were stored at 
\[ -70^\circ \pm 20\% \text{ (v/v) glycerol in horse serum, or as monolayer cultures at } 31^\circ \text{ with weekly medium changes, in which case subculture was necessary every 6–8 weeks. Screw-capped bottles (20 oz. medical flats, United Glass Bottles Ltd.), with a culture area of } 75 \times 160 \text{ mm., were preferred to 1 l. Pyrex Roux flasks with bungs for their ease of handling and cheapness; the glass is slightly 'alkaline' but is flatter than the available Pyrex. On average the bottles were used ten times and then rejected. Medium CSV.6 was also satisfactory for stationary bottle culture, giving yields of up to } 10^6 \text{ cells/ml., and a tenfold cell increase per week without medium change if inoculum and initial pH were correct (see below); use of } 0.1\% \text{ bicarbonate, } 0.1\% \text{ glucose and a less alkaline starting pH gave a faster growth rate (20-fold per week) but required a mid-week medium change. Routinely } 50 \text{ ml. of medium were added per bottle (initial pH } 6.7, \text{ equilibrating without gassing to pH 7.4) followed by } 4 \pm 0.2 \times 10^6 \text{ cells, and the bottles were left at } 37^\circ \text{ for 7 days before harvesting with } 2.5 \text{ mg. trypsin/ml. (Difco) in phosphate-buffered saline (Dulbecco & Vogt, 1954). Two separate lines were maintained, and some bottles were placed at } 31^\circ \text{ without subculture in case of loss by contamination. Yield was } 2-5 \times 10^7 \text{ cells/bottle. Bottles when empty were autoclaved in } 2\% \text{ (v/v) saturated solutions of sodium hexametaphosphate and sodium metasilicate in tap water, mechanically brushed with brushes made specially for these bottles (Matburn and Co. Ltd., London, W.C. 1), rinsed inside and out with cold tap water, } \text{n-HCl}, \text{ tap water again and finally four changes of distilled water before drip-drying. Care was taken to prevent drying before the final stage. It is believed that this is one of the simplest effective procedures; the alkaline treatment in time caused a 'bloom' on the glass which hindered cell growth and caused bottles to be rejected. Individual 'toxic' or 'greasy' bottles were rejected forthwith; routine rejection of a cheap bottle was preferred to varying the washing procedure.}

Operation of culture machine. The entire apparatus was autoclaved containing a few ml. of water for } 1\frac{1}{2} \text{ hr. at } 10 \text{ lb. in.}^{-2} \text{ in two parts, then mounted on its stand and the sterile connexion made (Fig. 1, S). Medium (20–40 l.) was pumped through a sterile filter-press via a sterile connexion (comprising an aluminium dust cap, Fig. 1, P', fitted inside with a short length of silicone tubing) into the reservoirs, and enough medium was run into the culture vessel to overflow. Four to six } 20 \times 20 \text{ cm. Carlson filter pads (EKS grade) were used and were pre-washed with } 8 \text{ l. distilled water and } 1 \text{ l. of medium; finer grades than this sometimes gave media yielding low growth rates. When filtration was finished, in order to avoid a liquid bridge the medium in the inlet line was drained out before disconnecting the filter press by opening the screw clip leading to the reservoir air-space. The stirring speed was increased almost to frothing, and the gas mixture started at } 10\% \text{ CO}_2 \text{ and } 15\% \text{ O}_2 \text{ and left overnight to equilibrate. The cells trypsinized from 20–40 bottle cultures were added next day to give } 4–6 \times 10^5 \text{ cells/ml. in the culture vessel, and were followed by mycostatin to give } 25 \text{ u./ml. The pH was controlled by}
varying the CO₂ content of the gas phase. Cell concentrations were determined by haemocytometer count on four replicate samples of 30–200 cells each in the presence of 2 mg. trypan blue/ml., blue-staining cells being classed as non-viable. With the chemostat, the cell count and the pH (as described below) were followed on a daily sample, and the gas and medium flow rates were adjusted daily to maintain optimum conditions; gas bubbles had to be dislodged from the pipelines. The turbidostat and chemoturbidostat, once set at optimal growth rate and cell density, needed a daily check on volume through-put and operation of components and a less frequent cell and pH check; cells in the receiver were fed twice-weekly with 1–2 l. medium. The pH was affected by both CO₂ and O₂ levels in the gas phase as well as by growth rate and cell density. Cells were harvested once-weekly from the receiver via the lowest port, and in order to avoid a liquid bridge the medium in the lower part of the port was drained out by opening the screw clip leading to the gas outlet. Occasional difficulty was found with the gradual formation of very large clumps which did not overflow from the culture vessel; these were siphoned off when necessary.

**Cell strain.** A transformed line of embryo rabbit kidney (ERK) cells was used. This was isolated by Westwood, Macpherson & Titmuss (1957) and grew well in medium CSV.6 after one or two subcultures in this medium in our laboratory.

**Cell state.** The microscopical appearance of the cells was a useful indication of culture conditions. Oxygen lack or low pH led to a granular cytoplasm and irregular outlines with a tendency to form syncytia; slight oxygen excess gave large clear well-defined cells, becoming very large in dangerous oxygen excess. Trypan blue staining was a good qualitative indication of cell viability (quantitative correlations were not made), but cells generally became trypan blue-positive only when it was too late to remedy the damage. A disadvantage was that the cells often grew in clumps; on a few occasions these became very large (c. 1 mm. diameter).

**RESULTS**

**Batch growth of ERK cells in stirred suspensions**

Many reports now describe growth of animal cells in suspension, and this was readily confirmed by us (Fig. 4) using a simple stirred pot gassed with 5% (v/v) CO₂ in air. These experiments showed that the cells grew rapidly on occasion but growth rate fell markedly above c. 2 x 10⁶ cells/ml., and the maximum density obtained was 5 x 10⁶ cells/ml. This limiting density depended on the surface area for a given volume of medium. Addition of glucose and arginine plus inositol (McLimans, personal communication) or a medium change sometimes but not always restored growth. Cell growth rarely started well below 2 x 10⁵ cells/ml. It was therefore clear that the optimum growth range for animal cells was narrow and was rapidly exceeded in culture, and unless good conditions were rapidly restored the cells entered a lag which was usually long or irreversible. Cell viability was very variable. Such growth also re-
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required daily attention if good yields were desired, and it was felt that a continuous culture, run under steady optimum conditions, would be more satisfactory. The following section describes attempts to obtain this steady state by adding medium at a constant rate ('chemostat').

![Graph showing rate of growth of ERK cells in batch suspension culture.](image)

**Fig. 4.** Rate of growth of ERK cells in batch suspension culture. Changes in volume indicate supplements of fresh medium or complete medium changes (arrows).

*Growth of ERK cells in suspension with continuous medium flow ('chemostat' principle)*

Fig. 5a shows the results of the longest of several runs in which growth was continuously maintained by uninterrupted medium flow. Herbert, Elsworth & Telling (1956) have shown that this type of system with bacteria is much more efficient than batch culture; this seems to apply also to animal cells. Over a period of 4 months, a culture-vessel volume of 1.5 l. yielded an average of $10^6$ cells per day in 1 l. of medium; this was a concentration rarely reached in bottle cultures with the same medium and a rate of yield not obtained in batch suspension culture. The maximum cell density was $2 \times 10^6$ cells/ml.; cell viability was 90–95%. The growth rate was also high, as the doubling time averaged 25 hr. over the whole period and was occasionally less than 16 hr.

Figure 6 shows that when cells were removed from the culture vessel and chilled, growth recommenced almost synchronously with a very short doubling time (14 hr.) if conditions were optimal. Most suspension cultures probably started synchronously, presumably because the cells cool during harvesting.

Unfortunately, although continuous medium flow was more efficient than batch growth, it was more troublesome to operate because the system was not self-balancing and not, therefore, a true chemostat although set up on the chemostat principle. As with bacteria, the cells were washed out of the culture vessel if a constant flow rate exceeded the growth rate; if the growth rate exceeded the flow rate, however, the cell concentration increased to a limiting value (usually with a very low pH) and the cells then entered a lag (i.e. growth was not restored by immediately restoring conditions to optimal values) so that the flow rate became excessive and the cells were rapidly
Fig. 5. A comparison of the yields and culture-vessel contents in continuous suspension culture of ERK cells during: a, a typical chemostat run; b, a typical turbidostat run; and c, a typical chemoturbidostat run.

Fig. 6. Synchronous growth from cells adapted to grow in suspension. Cells from the culture vessel during the run shown in Fig. 5(a) were centrifuged, shaken gently at 0° for 1-5 hr. and diluted in 100 ml. medium CSV.6 at 37° in a stirred and gassed culture vessel. Gas mixtures (v/v) were 9 % O₂, 5 % CO₂ and 86 % N₂ (Expts. O, △, ●) or 12 % O₂, 5 % CO₂ and 86 % N₂ (▲).
washed right out. As mentioned below, oxygen tension in the liquid phase (governed by oxygen tension in the gas phase and cell concentration, other factors being constant) also critically affected growth rate. It is possible that the culture would reach equilibrium if left to itself, but the narrow growth ranges, particularly of cell concentration and pH, make this unlikely; any attempts to allow equilibration were soon terminated as the culture would clearly soon be lost. Therefore, it was necessary every day without exception to anticipate the needs of the culture in the following 24 hr., and make adjustments accordingly. On two occasions a steady state was kept for some days, probably because oxygen tension happened to restrict growth rate to the same value as the flow rate.

We therefore conclude that with medium CSV.6 and ERK cells, which do not completely oxidize a large proportion of the available glucose, a culture machine running on the 'chemostat' principle is not a very satisfactory system. The main reason for this is probably that the cells can use glucose at a very high rate whether they grow or not, and glucose restriction does not much restrict growth without harming the cells. Other factors may contribute, however, particularly the difficulty of maintaining cell concentration, oxygen tension and pH in their narrow optimal ranges. Possibly a differently balanced medium or a rate-limiting component can be found which when deficient will slow growth without damaging the cells; meanwhile, a better self-regulating system was desired, and the approaches to this are described in the following section.

**Growth of ERK cells in suspension with automatically controlled medium flow**

Three methods of automatically controlling medium flow seemed feasible, based on (a) redox potential, (b) pH, (c) cell density.

**Redox potential.** Since growth rate depended on oxygen tension, growth should always be optimal if the oxygen tension or redox potential in the culture was poised at the optimal value. Two experiments were therefore conducted with an immersed platinum electrode and an isotonic saline-agar bridge leading to an external saturated KCl-calomel electrode. The e.m.f. of this cell measured with a pH meter was c. +200 mV. with medium only, but was unchanged by the addition of cells, whatever their state of growth. Presumably either the electrode polarized easily, or the high redox potential inside these cells (Cooper, 1959) does not encourage development of the very reducing environment encountered with bacteria (Hewitt, 1950). This method was therefore abandoned for control purposes.

**pH.** It proved feasible, using a demountable glass electrode (no. GSE. 23, Electronic Industries Ltd., Richmond, Surrey) to mount a glass electrode, with an internal platinum electrode but without the internal buffer solution, inside the culture vessel before autoclaving. After autoclaving the entire apparatus and allowing it to cool, the internal buffer solution was passed via a very fine silicone rubber tube, previously inserted, into the inside of the glass electrode. Using the same agar bridge as before, the system was calibrated
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for pH by measuring the e.m.f. of the glass-calomel pair when the medium in the culture vessel was without cells and in equilibrium with its gas phase (after 24 hr.). A sample of culture-vessel medium was then withdrawn, and while still at 37° gassed with the gas effluent from the culture vessel in a small non-sterile assembly which contained glass and calomel electrodes, themselves previously calibrated with standard buffer. The pH meter recording the pH in the culture vessel was then set to the true pH as determined on the withdrawn gassed sample.

This method showed that the pH of very recently withdrawn samples before gassing was about 0.1 pH unit higher than after gassing, and that visual estimates of pH from the phenol red indicator and with pH test-papers (British Drug Houses, Poole) gave agreement within 0.2 pH. The pH was routinely determined either with a pH meter or visually and with test papers on samples, but before opportunity for cooling and CO₂ loss had occurred. Cells briefly tolerated pH ranges of at least 6.6 to 7.4, but did not grow well unless within pH 6.9 to 7.1. This confirms the findings of Kuchler & Merchant (1956) with growth in suspension; estimation of the true pH near the cells is difficult in stationary bottle culture, where the pH in the bulk of the medium is about pH 7.3 for optimal growth. Use of the controller-recorder showed that a small alteration in the CO₂ content of the gassing mixture caused a detectable pH change in a few minutes, but the new pH value was not established fully for about 6 hr.; the half time was about 1 hr.

Once calibrated, the internal electrode accurately reflected changes in pH of the culture for several weeks at least. However, for simple measurement of pH this method had several disadvantages; (a) re-calibration was lengthy and was necessary 2 or 3 times a week; (b) initial assembly was difficult, particularly in obtaining satisfactory agar-bridges, since it was felt necessary to keep the saturated KCl from immediate contact with the culture; (c) breakage rate of glass electrodes was high; (d) while one electrode ran for 1600 hr. without trouble in the hands of Callow & Pirt (1956), others were less satisfactory. Since, as mentioned below, long runs are essential for our purpose (preferably > 1600 hr.) it is essential that any control system either be very reliable, or be external for replacement without stopping the culture. Its disadvantage as a control system was that maintaining the pH at 7 units was not by itself sufficient to promote rapid growth; this coupled with difficulty of operation led to our abandoning the immersed glass electrode both for pH estimation and growth control.

Cell density. Photo-electric control of the turbidity of a cell suspension (turbidostat) works well for bacteria (Bryson, 1952), and a similar system was accordingly applied to the apparatus described in Fig. 1. The principle is that an increase in cell density caused by growth increases the turbidity of the culture above a pre-set value and this automatically causes fresh medium to flow into the culture. The cells are thereby diluted until another pre-set turbidity value is reached, when the medium flow is automatically switched off again. Small mains voltage variations gave large variations in light intensity, and failure of the bulb or lighting circuit inactivated the control, or left the
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solenoid valve open so that medium flowed unrestrictedly; these difficulties were met or minimized by a double-photocell system, a multifilament bulb and the circuits shown in Fig. 3. Some cells settled on the glass over the photocell, but these were removed when necessary by scraping with the magnetic stirrer moved by a magnet held in the hand.

Two machines ran for 80 and 85 days respectively as turbidostats and the total cell production is shown for one (Fig. 5b). As may be expected, culture vessel contents were more stable than in the chemostat but the control was affected by non-viable cells, size of cells, and the turbidity which forms with stirring in absence of cells. Cell viability was 80–90%, in general somewhat less than with the chemostat. When conditions were optimal the growth was quite rapid, but the cultures recovered from periods of suboptimal conditions with difficulty and the cell density tended to drift to low values.

This revealed one fundamental disadvantage of the turbidostat as a system for culturing micro-organisms; unlike the chemostat, it has no intrinsic ‘incentive to growth’, and relies entirely upon one addition of medium producing sufficient growth to stimulate a second addition. The cell concentrations desired were close to the maximum supported by the medium, and these cells can use much glucose and possibly other substrates without necessarily growing; if in lag or suboptimal conditions, or in high cell density, they may not grow sufficiently to stimulate the mechanism before their glucose supply is exhausted, and the culture will be lost unless detected in good time and given a supplement of glucose, or a medium change, or deliberately diluted. Thus a pure turbidostat could be used but was not entirely satisfactory; the following section describes a combination of both chemostat and turbidostat principles.

The chemo-turbidostat

The chemostats in general gave good growth, and their main disadvantage was that literally daily attention was necessary and an error in judging in advance the medium flow rate in relation to cell growth rate sometimes rapidly lowered the cell density below levels permitting growth, or increased it to values at which the cells entered a lag phase. Therefore, several chemostat runs were made in which this snag was met by using the turbidimetric control to stop the supply of medium if the cells were diluted below a certain pre-set minimum. For this, medium was continuously passed in small intermittent doses by means of a time switch actuating the solenoid closure, at a rate if anything slightly greater than the growth rate. The photocell arrangements of Figs. 1 and 2 and the circuits of Fig. 3b or c were used.

Such a system appeared fairly satisfactory, combining ‘incentive to growth’ with a reliable safety device, and requiring only a rough initial balancing of growth rate and medium input and much less routine attention. Fig. 5c shows a run with this system, additionally using a fructose- or galactose-glucose carbohydrate source. Its cell-production rate averaged the same as the turbidostat but was more stable.
Variation of carbohydrate source

A difficulty in the continuous running of cell-culture machines with the ERK cell was the large amount of non-volatile acid produced. Eventually the cultures often became too acid for growth even with no CO₂ in the gas phase, necessitating a medium change or addition of bicarbonate. It was hoped to avoid this by replacing glucose by galactose (Eagle, Barban, Levy & Schulze, 1958), but four attempts (two in stationary and two in continuous suspension culture) failed to subculture cells indefinitely in CSV 6 containing galactose in place of glucose; the cultures died after 10–20 generations. However, fructose alone, or a mixture of galactose or fructose (6 g./l.) and glucose (2 g./l.) appeared to carry the cultures well with good growth and less production of non-volatile acid.

Receiver conditions

As it was convenient to harvest the cells only once-weekly, some storage system was needed. Efficient running would entail a depletion of the environment in the culture vessel, so that without attention the cells overflowing and collecting in the receiver may be in poor condition after 7 days. This was found to be so, and was remedied in part by maintaining the receiver at 30°, and supplementing with fresh medium as follows: after harvesting and draining out all cells, 1–2 l. of fresh medium were run into the receiver from the reservoir as an initial feed, followed by 1–2 l. on the third day and a further 1–2 l. on the sixth day, the cycle being completed by harvesting on the seventh. Viability was then about the same as in the culture vessel. This method, however, is not entirely satisfactory and there is evidence that the cells obtained are not in as good condition as rapidly growing ones; this aspect therefore requires more study. The cooling was insufficient to stop growth completely so that some additional cells were produced by growth in the receiver; sometimes overall yield was deliberately increased by large feeds and by increasing the receiver temperature, but this expedient was inefficient.

The effect of oxygen tension on growth rate

Fig. 5a shows that the culture vessel content during the chemostat run varied widely from day to day; this was because the growth rate also varied. As may be expected, one factor affecting growth rate was the gas phase oxygen tension; growth was slowed by both oxygen lack and excess, the latter sometimes occurring at concentrations less than atmospheric. On two occasions during nearly steady states (Fig. 7) a small increase in oxygen concentration (from 22 to 24%, v/v, at day 37) halved the growth rate, and a small decrease (from 19 to 17% at day 45) increased the growth rate by 50%. In other experiments higher concentrations (25–30%) were rapidly cytocidal.

Presumably the important factor is the O₂ concentration in the liquid rather than the gas phase, and this will depend on cell concentration among other factors. Figure 7 gives the relations between growth rate, oxygen concentration and cell concentration during two portions of the chemostat run shown in Fig. 5a, and shows that growth rates frequently passed through maxima in a
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cyclic manner. A plot of cell concentration against oxygen tension for the 24 hr. periods during this run when growth rates were maximal (Fig. 8) shows that the two are related, and the extrapolation to zero cell concentration suggests that the optimal oxygen concentration in the liquid phase is equivalent to equilibrium with about 9% (v/v) oxygen in the gas phase. This concentration is probably not absolute, however, and may, for example, depend on the catalase content of the serum (Lieberman & Ove, 1958), but the curve was useful in selecting oxygen tensions for optimal growth at given cell densities. Since the cell concentration varied, the liquid-phase oxygen tension should also vary, and this variation presumably caused the changes in growth rate since other conditions were steady; as growth rate in turn affected cell concentration, it is not surprising that conditions were cyclic.

![Graphs showing growth rate and culture-vessel content](image)

Fig. 7. Abstracts from the chemostat run of Fig. 5a, showing effects of abrupt oxygen gas-phase changes during steady state (arrows) and cyclic response of growth rate and culture-vessel content.

Fig. 8. Relation between gas-phase oxygen tension and average culture vessel cell content for all periods when the daily percentage cell increase exceeded 87% (probable mean doubling times of 24 hr. or less) during the chemostat run shown in Fig. 5a.

Osgood & Krippaehne (1955) reported that cells on an inclined immersed microscope slide grow best 1–2 cm. below the surface. Apart from this, the gas phase of tissue cultures has been relatively neglected, but several authors have recently reported that oxygen can be in inhibitory excess at quite low concentrations (Cooper, Burt & Wilson, 1958; Zwartouw & Westwood, 1958; Lieberman & Ove, 1958). Oxygen tensions are also low in tissues in vivo (Davies & Bronk, 1957). Lieberman & Ove (1958) state that the oxygen toxicity, particularly marked with single cells, can be overcome with catalase. The inhibitory effect of oxygen may be complex, however, and may perhaps only apply if cells are partly anaerobically adapted; for example, cells in roller tube culture are probably highly aerobic most of the time, yet grow well.
DISCUSSION

The term 'continuous culture' is often applied to growth of micro-organisms with continuous medium flow, but is also sometimes applied to batch growth of animal cells when unlimited subculture is achieved. We assume unlimited subculture and use 'continuous culture' for continuous medium flow with suspended cells (chemostat or turbidostat), 'batch-culture' for discontinuous culture of suspended cells and 'bottle-culture' for the necessarily batch growth of monolayers on glass. The terms 'deep' and 'submerged-fermentation' are used in the fermentation industry to distinguish suspended culture from culture on or near the liquid surface; although occasionally used for suspension cultures of animal cells, these terms are avoided here as the distinction does not apply to our system. It should be noted also that for convenience we use the term 'chemostat' for an apparatus set up on the chemostat principle, although in practice it rarely functioned as such.

Several different types of assembly have been used for successful batch-culture of suspended animal cells (Graff & McCarty, 1957; references in Ziegler et al. 1958). In starting the present work we accepted the principle that the simplest system is the stationary 'stirred-pot'. This is general in the fermentation industry; particular advantages are its ease of construction and scaling up or down.

A second principle is that continuous culture can be more efficient and less troublesome than batch-culture (Herbert et al. 1956). We modelled our system where possible on those satisfactory for bacteria (Herbert et al. 1956; Elsworth, Meakin, Pirt & Capell, 1956), while using ordinary laboratory equipment and allowing for differences in the cells used, animal cells being (a) more easily sedimented, (b) physically more fragile, (c) more sensitive to adverse chemical environment and (d) much slower growing than bacteria. For the last reason, and also because of the complexity of the medium, no theoretical studies were attempted and we assumed that volume change and growth rates at steady state are related as for bacteria (Herbert et al. 1956). As an example of the difficulties of theoretical work, Herbert et al. allowed their bacterial cultures 24 hr. to equilibrate after changing conditions slightly; an equivalent time for animal cells would be more than one month.

A third principle is that the apparatus must be reliable in every sense. Where possible, the cheapest and simplest laboratory apparatus has been used, except where apparent simplicity may affect its continuous running. This is because small mechanical failures were often lethal to the cells, and chance contamination has not been countered by any means except dismantling and re-sterilizing, except in two cases where small moulds found growing inside the reservoirs were completely inactivated by adding mycostatin (500,000 u./20 l.). Since dismantling, cleaning and re-sterilizing took several days, growth of a fresh inoculum (10⁹ cells) sometimes took a week, and the cultures once started took 1–2 weeks to reach high yields again, the system was uneconomical unless at least 2 months continuous operation could be expected. However, sources of unreliability were eliminated once found and the components
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described have been quite reliable; the main chance of breakdown lay in contamination during inoculation or replenishing medium in the reservoirs. Harvesting and sampling were adequate with the devices described. Much protection was probably afforded by the antibiotics; however, because of its higher growth rate, once an antibiotic-resistant variant appears the contaminant will be much more likely to take over the culture than in bacterial chemostats (Powell, 1958).

Thus with continuous culture of animal cells, if one needs a large yield one also needs a relatively complex assembly, but a smaller-yielding apparatus could be much more simple. It is likely that the cell needs of a small diagnostic or research laboratory could be met by a small apparatus with a culture-volume of 300 ml., a simplified chemo-turbidostat (Fig. 3c) and a slow gas supply (10 ml./min.) from a 5% (v/v) CO₂-air cylinder. This should run for several months with little attention, although it seems unlikely that the yield-rate would be optimal. On the other hand, the large cell needs of a commercial plant producing vaccines might well be met by one 10 l. capacity fermentor of a design commercially available. The virus production by these cells will be considered elsewhere, but a single fermentor should be able to match the poliovirus output of most vaccine organizations if the objection regarding possible malignancy of cells were overcome; this objection may not apply to agricultural animal virus vaccines. A more economical medium than CSV. 6 could almost certainly be found.

In practice, when differences in cell concentrations were allowed for, the yield rates of the three types of assembly described differed by less than two-fold, although the actual yield of the chemostat arrangement was greater than the yields of the automatically controlled devices, partly because the attention the former demanded ensured that conditions were more often better and because the latter tended to drift towards lower cell concentrations. Despite this, however, an automatically controlled chemo-turbidostat is much to be preferred for its ease of operation; present work is directed to increasing practical culture-vessel cell populations.

We are indebted to the National Fund for Poliomyelitis Research, by whose generosity the Virus Culture Laboratory was established and this work supported. We are also grateful to Dr F. Sheffield for the original gift of the ERK cells, and to Dr E. Klieneberger-Nobel for testing our cultures at intervals for pleuropneumonia-like organisms.

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A SENSITIVE AND ACCURATE PLATE ASSAY FOR POLIOVIRUS NEUTRALIZING ANTIBODY

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Assay of neutralizing antibody by end point tube dilutions is not very accurate unless large numbers of dilutions are used, and neutralization of plaque counts (1) also requires many manipulations per sample. Similar difficulties were solved for antibiotic assays by the use of a cup plate gel diffusion method (2); such a method for neutralizing antibody should have wide application because of its accuracy and ease of use for many samples at one time. Some such methods have been described, based essentially on monolayer plaque assays (3, 4, 5). The present paper describes a method based on the agar cell-suspension plaque assay (6) which is as accurate as the plaque neutralization method, yet gives neater zones and is more sensitive than monolayer gel diffusion methods.

MATERIALS

Petri dishes. Only flat Petri dishes were used, either 4-inch size (90 mm internal diameter) Czechoslovakian (Anumbra) glass (Travis & Co. London, E.C. 1) or 6-inch size (135 mm internal diameter) plastic phage typing dish (PD 15025 PC, Falcon Plastics Co., Culver City, California). The plastic dishes can be re-used after standing overnight in a mixture of equal volumes of sodium hypochlorite and saturated sodium hexametaphosphate and sodium metasilicate solutions, rinsed well in glass distilled water and sterilized under an u.v. lamp.

Medium. For most of this work the agar medium contained 1.25% washed Difco agar and modified Earle's saline medium as described for plaque assays by Cooper (7). Alternatively, the galactose medium (7), where galactose replaces the need for glucose and CO₂-bicarbonate buffers, was equally effective although staining was less intense.

Antisera were made in donkeys to poliovirus types I, II and III and kindly donated by Dr. John Beale, with tube end point neutralization titers given as 1:5,000 to 1:10,000.

Poliovirus stocks were type I Brunhilde, type II MEF₁, and type III Leon grown in embryo rabbit kidney (ERK) cells.

Cells used were a continuous line of ERK cells (8), grown as described by Cooper et al. (9). The cells used for the agar cell-suspension assays were obtained either from freshly trypsinized monolayers or from cells conserved in a stirred suspension for 1 to 2 days at 31°C (7). In either case the cells were washed in fresh growth medium and resuspended in medium at 10⁷ cells/ml. When they were not needed immediately, the cells were kept at 0° to 4°C on a rocker to avoid clumping and anoxic effects. Providing the viability as given by trypan blue staining of the conserved cells was over 80%, there was no notable difference in results using cells from either source.

METHODS

The method recommended is the agar cell-suspension modification; two versions of this are given in which the antiserum is applied to agar base layers either in cups cut in the agar or in “fish-spine insulator beads” (10). These versions have slightly differing merits. A monolayer method, modified for maximum sensitivity, (but otherwise similar to those described by De Somer and Prinzie (3) and Porterfield (4)) in which the antiserum is applied either in filter paper disks or in fish-spine beads to monolayers with prepoured agar overlays, was used for comparison.

Agar cell-suspension cup plate

Fifteen milliliters of molten agar medium were poured into 4-inch (or 50 ml into 6-inch) Petri dishes, allowed to set on an accurately leveled glass plate and stored for 2 hr at 4°C to harden. The cups were put by a stainless steel tube with one end beveled internally to cut a hole of 8 mm diameter; seven cups were cut in 4-inch dishes and 16 in 6-inch dishes. Equal quantities of serum
dilution and molten agar medium containing 2% agar were mixed and 0.1 ml of this mixture was pipetted into the cup. When all cups were filled, the plate was placed at 4°C in humid air containing 5% CO₂ for 24 to 72 hr to allow diffusion, and then overlayed with a mixture containing cells and virus and an equal volume of molten medium containing 1.25% agar. The 4-inch plate required 10⁶ cells + 10⁶ plaque forming units of virus in a total volume of 2.5 ml of agar overlay, and the 6-inch plate 3 × 10⁵ cells + 3 × 10⁵ plaque forming units in 6 ml. The agar overlay was allowed to set on a leveled surface, and dishes were incubated at 37°C in a 5% CO₂-air mixture for 48 to 72 hr and then stained with 2 ml of a tetrazolium salt (2-p-iodophenyl-3-p-nitrophenyl-5 phenyl tetrazolium chloride, 1.5 mg/ml in a solution containing 9 mg sodium chloride/ml and 20 mg glucose/ml (11)).

Agar cell-suspension bead plate

The base layer was poured according to the previous method but with 7.5 ml agar medium in a 4-inch dish and 15 ml in a 6-inch dish, and set for 30 min at room temperature. Sample dilutions of serum were prepared and aliquots placed in the cups of a plastic agglutination tray. Size 3 fish-spine insulator beads (Taylor, Tunnicliff & Co. Ltd., Stoke-on-Trent, England), prewashed in chloroform to remove grease and boiled in glass distilled water, were touched on the surface of the sample dilution and gently placed on the agar, using sterile forceps. Seven beads were used on 4-inch and 16 on 6-inch dishes. After diffusion as above, the beads were removed and the plate was placed, open, at 37°C for 1 to 2 hr to dry. The pouring of the overlays and the subsequent treatment were as described for the previous method.

Monolayer disk and bead plates

Only 4-inch dishes were used as the cells did not form monolayers on the 6-inch plastic dishes. A confluent monolayer was grown by placing 2.5 × 10⁶ cells + 10 ml medium in a Petri dish and incubating at 37°C in 5% CO₂-air mixture for 24 to 36 hr. After the medium was decanted, 2 ml of molten agar medium were allowed to set in each plate and the beads or filter paper disks placed on as described above. The dishes were replaced in the CO₂ incubator at 37°C for 24-hr diffusion. The beads or disks were then removed and the dishes left to dry for 2 hr. A further overlay containing 10⁵ plaque forming units of virus in 3 ml molten agar mixture was added and the dishes were incubated in CO₂-air mixture at 37°C for 48 hr, when they were stained as before.

RESULTS

Relation between serum concentration (dose) and neutralization zone

All three methods gave good circular neutralization zones, with fairly sharp edges and strong stain contrast (Figs. 1 and 2).

The results have generally been plotted as the diameter of the neutralization zone against the logarithm of relative serum concentrations, and have yielded straight lines (Figs. 3 to 5). De Somer and Prinzie (3) and Farrell and Reid (5), using disk monolayer plates, found a linear relation between zone diameter and logarithm of concentration, whereas Porterfield (12) using bead monolayer plates found a quadratic relationship. We find that either a plot of diameter or of the square of the diameter against logarithm of concentration gives equally adequate straight lines; in fact, it has been pointed out that, although the relationship should in theory be quadratic, the

![Figure 1. Neutralization zones formed by serial 2-fold dilutions of type 3 poliovirus antiserum in an agar cell-suspension bead plate. The largest zone is formed by a 1:4 (final) dilution of antiserum, and the smallest (trace in the center of the plate) by a 1:256 dilution, equivalent to an end point dilution titer of approximately 1:20. Three days prediffusion at 4°C, 2 days incubation at 37°C.](image)
coefficient of variation of the assays would have to be very low to differentiate between the two types of relationship (10). We, therefore, do not regard the difference as significant and use the linear plot for convenience.

Factors affecting sensitivity

The highest sensitivity was required, that is, the largest clearly defined neutralization zone for a given serum dilution. The following factors were examined for their effect on sensitivity.

Diffusion times. A major advantage of the agar cell-suspension methods over the use of monolayers was that antiserum could be allowed to diffuse into the base layer for several days before the cells were added, thus giving much larger zones and therefore a higher sensitivity (Figs. 4 and 5). Attempts to diffuse antiserum into agar-covered monolayers at 0°C overnight, or for longer than 24 hr at 37°C, produced much cell destruction. If necessary, zones could be obtained quickly by the cup plate suspension method using 24 hr diffusion before adding the cell overlay and staining after a further 2 days, but sharper contrast of staining and larger zones were obtained by diffusing for 72 hr and staining after a further 72 hr. With cell suspension bead plates the minimum time for diffusion was 48 hr, but 72 hr was optimal. Drying the plates at 37°C for 1 to 2 hr after removing the beads prevented smudging of the zones when the overlay was poured.

Normally all diffusion was allowed to occur at 4°C, in order to minimize inactivation of medium
Figure 4. Comparison of sensitivities of the agar cell-suspension (O) and monolayer (●) bead methods, using the same dilutions of type 3 antiserum.

Figure 5. Effect of time of diffusion and sample holder (bead or 5-mm paper disk) on sensitivities, using the agar cell-suspension method and type 3 poliovirus. ● = bead, ▲ = disk; 72 hr diffusion before adding cell/virus overlay. O = bead, △ = disk, placed on overlay with no prior diffusion.
and chance contamination, and was usually carried out in a sealed box previously gassed with CO$_2$/air mixture, although the high pH occurring in air alone had no apparent effect. Galactose could with advantage replace glucose and sodium bicarbonate in the medium (7) as it avoided the pH changes associated with bicarbonate; the galactose medium gave results similar to the glucose-bicarbonate medium (Fig. 1) although the zones stained less densely.

**Antiserum in the base layer.** In an effort to increase the sensitivity of these methods, small amounts of antiserum were included in the base layer before addition of test samples for diffusion. The optimum concentration was about that which just inhibited plaque development when incorporated in the agar of ordinary plaque assays ($\frac{1}{400}$ of the serum used), and this increased the diameter of the zones by about 50% without affecting the accuracy of measurement, and decreased to about one third the antiserum concentration at which zones were just detectable. The effect was probably not equivalent to inoculation with less virus, since this gave larger but more ragged zones, with correspondingly less accuracy. It is more likely that an equal number of cells were infected in the first cycle but the effective yield per cell was reduced; in other words, the total number of microplaque was the same but they developed more slowly. To conserve serum this device was not used routinely but only when maximum sensitivity was needed.

**Volumes of agar.** For agar cell-suspension cup plates, the agar base layer thickness used was necessary to accommodate 0.1 ml of sample in the cups. The volume for the cell-virus overlay was the smallest practicable. For agar cell-suspension bead plates the agar base layers were also minimal in order to produce the largest possible diffusion zone, which increased with decrease in agar thickness. The cell-virus overlay on these plates and the virus overlay on the monolayer bead plates were similarly reduced to a minimum.

**Cell number.** The number of cells giving maximal sensitivity was $10^9$ per 4-inch dish, (slightly more than that usual for plaque assay plates (7)), and $3 \times 10^9$ on the 6-inch dish. More cells gave smaller zones, whereas fewer cells plus less virus gave the same size zones which were, however, fainter and less accurately measured. Cell number in monolayers was restricted to those which gave confluency (5 to $10 \times 10^6$).

**Cell-virus ratio.** Using $10^9$ cells in a 4-inch dish, a dose of $10^8$ plaque forming units per dish of all three serotypes of poliovirus was optimal for both the agar cell-suspension cup and bead plates. Less virus gave larger zones which were irregular in shape and difficult to measure because of ragged edges. More virus reduced zone size and intensity of staining.

**Size of serum container.** As the containers were relatively small, the sensitivity increased with the volume of serum which they contained. The 5-mm filter paper disks (3), containing 0.02 ml, were less sensitive in our hands than the fish-spine beads (0.04 ml); larger disks (3⁄4 inch, containing 0.13 ml, (5)), should be more sensitive still, as were the cups-in-agar (0.1 ml).

**Factors affecting accuracy**

The most important factor affecting accuracy was found to be the constancy of depth of the agar over the surface of the plate; varying depths gave noncircular zones with poor agreement between duplicates. Hence the need for the optically flat glass and plastic dishes recommended, and also a leveled 3⁄4-inch glass plate for pouring the base layers.

Zones were measured with dividers to the nearest 0.5 mm taking the average of two diameters at right angles. Preferably four replicate zones were included, two on each of two plates, and the average diameter was calculated. Usually the maximum difference between diameters of replicates was 20% of the mean in the range 8 to 25 mm normally used. The coefficients of variation in the three six-sample experiments in Figures 3 and 5, each representing essentially 12 replicate determinations of the same sample, were 21%, 14.8% and 17.9%. The reproducibility should be equivalent to this accuracy, since standards must be included.

**Discussion**

The agar cell-suspension gel diffusion method for antibody assay was an accurate, rapid and moderately sensitive method for screening large numbers of antisera. Avoiding the use of monolayers increased sensitivity; the sensitivity of interferon assays (4) might also be improved by first allowing the interferon to diffuse in a base layer, then adding a layer of cells and allowing this to interact with interferon for an optimal time, and then adding a final layer of virus. Agar cell-suspension plaque assays have a number of other operational advantages over monolayer assays (6, 7).

Of the two agar cell-suspension methods, the
cup plate required more operations but was more
sensitive because a larger measured quantity of
antiseraum could be used and results could be ob-
tained in 4 days if necessary. The bead plate used
less material with less manipulations and was there­fore considerably more convenient, but it took longer; 5 days altogether was the minimum, and 6 days was usually used for best sensitivity.
The coefficients of variation found (15 to 20%) were similar to those of the careful plaque neu­tralization method described by French and co-
workers (1); plaque neutralization has the merit of
giving absolute values without relying on
standards but is subject to the usual statistical
limitations of plaque assays. The gel diffusion
methods are quicker and much more convenient
than plaque neutralization and roller tube end
point dilution, but are not so sensitive. Incorpora­
tion of low concentrations of antiserum in the base
layer appeared a useful device for increasing the
method's sensitivity.
As mentioned above, the main disadvantage of
the monolayer method was its 5- to 10-fold smaller
sensitivity, but it had the single merit of allowing
microscopic examination of cells for cytopathic
effect without staining. The use of 5-mm filter
paper disks to contain the antiserum (3) is less
convenient than fish-spine beads and is less than
one-tenth as sensitive, presumably because the
volume contained is less; larger disks (5) may
overcome this disadvantage. De Somer and
Prinzia (3) suggest that the monolayer-disk
method can detect end point neutralization titers
of 1:5; it is difficult to compare titers obtained in
different laboratories, but our data indicate that
similar titers might be detected (but not accur­ately measured) using the agar cell-suspension
cup plate assay containing low concentrations of
antiseraum in the base layer, provided that the
virus inoculum is very low. Using the higher virus
inocula needed for accurate measurement, how­ever, the lowest titers measurable are probably
around 1:30 to 1:50, and the method should
suffice for routine screening of recently vaccinated
individuals.
The main uses envisaged for the method are the
accurate comparisons of unknown with standard antiserum and the measurement of virus antigen concentrations by determining serum
titers before and after incubation with concen­
trated virus preparations (measurement of
"serum-blocking power"). For the former, 2-fold
dilutions of the unknown serum were placed on
a cell-suspension bead plate with equal numbers
of 2-fold dilutions of a standard serum; relative
titers were obtained by reading the zone diame­
ter of the unknowns for the dose-response curve
provided by the standard (similar to those of
Figures 3, 4 and 5) and averaged where necessary.
For measurement of serum blocking power, varying dilutions of poliovirus (concentrated 10-
fold by centrifugation from crude tissue culture
fluid which contained 10⁶ plaque forming units/ml, and then clarified by slow centrifugation) were
incubated overnight at 4°C with undiluted stand­
ard antiserum, and the residual antibody com­
pared on cell-suspension bead plates with dilu­	ions of the standard antiserum. It was found that
amounts of virus antigen equivalent to as little
as 10³ plaque forming units could be detected,
but as the antibody blocked per unit of virus
was not the same at all virus concentrations the
method required further investigation.
Acknowledgment. We are indebted to Dr. John
Beale for generous gifts of antiserum.

SUMMARY
A sensitive gel diffusion method is described
for the detection and assay of poliovirus anti-
era, based on extended diffusion of antibody
from agar cups or fish-spine beads in an agar
base layer before addition of an agar suspension
of virus infected cells. The advantages, sensitiv­
ity, and accuracy of this method are compared
with others in current use; it should be useful for
rapid screening of sera from recently vaccinated
individuals.

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Photodynamic Demonstration of Two Stages in the Growth of Poliovirus

When poliovirus is grown in the presence of neutral red (NR), the virus in the yield can be readily inactivated by exposure to visible light (1), an effect presumed to be due to incorporation of the dye in the mature particle. In the following one-step growth experiment, the virus inoculum was grown in the presence of NR in the dark, and used to infect cells in the presence of NR. It is shown that the light-sensitive inoculum produced an infective center which went through a transient period of light resistance before the light-sensitive progeny appeared within the cell.

The experiment was set up as follows. Petri dishes (5 cm diameter) were seeded with $4 \times 10^6$ ERK cells in 5 ml of medium CSV.6 (2) containing $4 \mu g$ NR/ml, and incubated for 16 hours at 35° in a dark box flushed with 5% CO$_2$ in air. This concentration of NR had little effect on cell or virus growth in the dark but almost totally prevented virus growth with the visible light irradiation described below.

After adding the NR, cells and resultant virus were handled and assayed strictly in darkness or under a dim safe-light (light from a 30-watt tungsten lamp filtered through 2 cm of a 20 $\mu g$ NR/ml aqueous solution). After incubation, the monolayers were drained, washed with phosphate-buffered saline, and completely infected by adsorbing virus for 10 minutes from 1 ml containing 22 plaque-forming units per cell of light-sensitive poliovirus type 1 (Brunners), itself grown in darkness in medium CSV.6 containing 4 $\mu g$ NR/ml. After infection, the monolayers were drained again and washed twice, 5 ml of CSV.6 containing 4 $\mu g$ NR/ml were added and the dishes were replaced in CO$_2$-air. The temperature was kept at 35 ± 0.1° throughout.

At intervals, one monolayer was removed from the CO$_2$ box and irradiated at 35° for 15 minutes (46 cm below an 84 cm, 80-watt "daylight" fluorescent strip) and replaced in the CO$_2$ box. The 15-minute pulse was sufficient to inactivate at least 95% of the inoculum virus, but had only a slight effect on the postirradiation growth in darkness of cells grown and maintained in 4 $\mu g$ NR/ml, and negligible effect on the growth of poliovirus in such cells when infected after irradiation.

![Graph](Image)

**Fig. 1.** The effect of a pulse of visible light when applied at various times after infection of neutral red-containing cells by poliovirus grown in neutral red. Curve A: Replicate ERK cell monolayers maintained in 4 $\mu g$ neutral red per milliliter were infected in darkness with light-sensitive virus, as described in the text. At the intervals after infection indicated on the abscissa, one monolayer was irradiated for 15 minutes and returned to darkness. At 8 hours after infection, when virus growth had ceased, all cultures were lysed and assayed under a safe-light for virus yield. Curve B: a one-step virus growth cycle carried out in darkness under conditions comparable to those of curve A, in which one monolayer culture was lysed with deoxycholate at the times indicated in the abscissa, and assayed for virus yield.
Eight hours after the addition of virus, all monolayers were lysed by the addition of sodium deoxycholate to 2 mg/ml and assayed for virus content by the agar cell-suspension plaque method (3), maintaining darkness or safe-light conditions throughout. The resultant yields are shown in Fig. 1, curve A; curve B shows a comparable one-step growth curve in which cells were lysed for virus assay at intervals during the growth cycle. It can be seen that irradiation during the period 1–3 hours after infection had little effect on the ultimate yield of these cultures, and that the yield was greatly reduced by irradiation before or after this period.

Thus, in spite of the continuous presence of NR, the poliovirus growth process is resistant to light during a portion of the growth cycle which coincides with the eclipse period, so that this finding seems likely to reflect postadsorption changes in the state of the infective entity at or about the times of penetration and maturation.

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P. D. Cooper
Early Interactions between Poliovirus and ERK Cells: Some Observations on the Nature and Significance of the Rejected Particles

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At temperatures above 23°, 60-80% of adsorbed P32-labeled poliovirus particles, apparently in combination with cellular material, were sloughed from ERK cells. Unlike virus, the sloughed labeled material adsorbed to diethylaminoethylcellulose, but not to cells; its density in a tartrate gradient was 1.20-1.22; it was split by crude trypsin and precipitated by ether. Its infectivity was not restored at pH 2.5. Sloughing was inhibited by 10 mM glutathione.

After adsorption to cells at 0°, full virus infectivity could be recovered with deoxycholate. At 20°, and probably still at the cell surface, adsorbed infective virus was reversibly inactivated: its infectivity could be restored at pH 2.5 but not by deoxycholate. At 20°, infective centers became resistant to antisera although sloughing of labeled particles was prevented. However, preincubation of infected cells at 20° neither reduced the amount of P32 subsequently sloughed at 37°, nor increased the number of cells infected. Most of the nonsloughed labeled material remained resistant to the action of ribonuclease for up to 2 hours after infection.

INTRODUCTION

Poliovirus growing in suspension cultures of ERK cells, after adsorption at 0°, provides a reproducible and precisely controlled system in which to study the processes of infection. With a high multiplicity of infection (when random delay is minimized) the infective centers become resistant to antiviral serum within a few minutes at 37° (Cooper, 1962), and the newly matured progeny reach a level of 1 plaque-forming unit per infected cell at about 2.7 hours after infection.

This paper describes some of the properties of that fraction of the adsorbed P32-labeled virus which is rejected by the cells (Joklik and Darnell, 1961) during the first hour after infection in the poliovirus-ERK cell system. An attempt has been made to assess the significance of this rejection in relation to infection and to some other processes which an infective particle may undergo shortly after adsorption to a cell.

MATERIALS AND METHODS

Media. Phosphate-buffered saline (PBS) was used for washing cells and adsorption of virus. Virus growth usually occurred in CSV6 medium (Cooper et al., 1959) or in a similar medium containing galactose in place of glucose, and without bicarbonate, called GCM (Cooper, 1961).

Virus assay. Infectivity was measured by the agar cell-suspension plaque assay of Cooper (1961) and is expressed as plaque-forming units (PFU) per milliliter.

Materials. Crystalline ribonuclease (RNase) was obtained from Armour Laboratories, glutathione and oxidized glutathione from L. Light & Co. Ltd., and diethylamino-
ethyl (DEAE)-cellulose (Whatman DE 50 powder) from H. Reeve Angel and Co. Ltd.

**Cells.** All experiments involved cells maintained in suspension. ERK cells grown in bottle cultures (Cooper et al., 1959) were trypsinized, suspended in medium CSV6 (5 × 10⁶ cells/ml), and stirred overnight at 37° while being gassed with 5% CO₂ in air. This incubation improved their ability to adsorb virus at 0°. They were used only if the proportion of nonviable cells (cells which absorbed stain from 2 mg trypan blue/ml) was less than 10%. The cells were washed with PBS before infection.

**Preparation of Pb²⁻labeled virus.** Type 1 (Brunenders) poliovirus was grown and purified essentially according to Hoyer et al. (1959), but using ERK cells in suspension culture. A batch of cells (5 to 10 × 10⁸) was washed with 0.14 M NaCl, suspended in Puck's medium (Marcus et al., 1956) containing no phosphate, 0.001 M citrate, 5% dialyzed horse serum, and 0.2% NaHCO₃, and gassed with 5% CO₂ in air. The suspension contained 10⁷ cells/ml, 3 × 10⁷ PFU/ml, and 30 µc carrier-free P³²O₄⁻/ml, and was stirred at 37° for 8 hours and then frozen to −20°. Virus yields were 50-100 PFU/cell. The tissue culture fluid was centrifuged for 15 minutes at 10,000 g and the resulting supernatant further centrifuged for 90 minutes at 120,000 g. The pellets were resuspended with vigorous pipetting in 0.02 M phosphate buffer (pH 6.8), and usually left overnight at 4°. The supernatants after a further centrifugation for 15 minutes at 500 g (about 4 ml) were applied to a DEAE-cellulose column (1-cm diameter, containing 1.2 g of DEAE-cellulose packed at a pressure of 1 pound per square inch), using 0.02 M buffer as eluent. The first peak of radioactivity, which passed straight through the column, contained the virus and was stored at 4° after adding NaCl to 0.14 M. Further centrifugation with or without a CaCl₂ or tartrate density gradient (see below) did not increase its specific infectivity (2 to 6 × 10⁴ PFU/cpm). A slight precipitate sometimes formed when material which had been treated with DEAE-cellulose was kept at 4°, and was removed by centrifugation without affecting the radioactivity or infectivity. The overall recovery of infectivity from the tissue culture fluid was generally 50-75%, the greatest losses occurring during the ultracentrifugation and resuspension.

**Preparation of S³⁵-labeled virus.** S³⁵ cysteine and methionine of high specific activity were prepared from Escherichia coli. The organism was grown for 16 hours at 37° from a washed inoculum, on agar medium containing (per milliliter): 1 mg NH₄Cl, 3 mg NaH₂PO₄, 1.5 mg KH₂PO₄, 1.5 mg NaCl, 0.01 mg MgCl₂, 2 mg glucose, 14 µg Na₂SO₄, and 100 µc carrier-free S³⁵O₄⁻. The bacteria were scraped from the agar, washed once with water and twice with 0.6 M trichloroacetic acid, and autoclaved for 1 hour in 6 N HCl; the hydrolyzate was evaporated to dryness and dissolved in water. The hydrolyzate from the yield from 50 ml of S³⁵ agar was added without further treatment to 25 ml of the medium for virus growth. The virus was grown and purified as described for P³² virus, but the medium lacked cystine and methionine instead of phosphate; after addition of the hydrolyzate, the medium contained about 100 µc S³⁵/ml and 5–10 µg each of cystine and methionine per milliliter. The DEAE-cellulose-treated preparation, obtained as with the P³² virus, was centrifuged for 90 minutes at 120,000 g and the pellet was resuspended in 0.14 M NaCl. The discarded supernatant contained about 50% of the S³⁵, but very little infectivity. The purified virus (specific infectivity 2 × 10⁴ PFU/cpm), when centrifuged through a tartrate density gradient, produced a single band of radioactivity, which coincided with the infectivity.

**Preparation of C¹⁴-labeled virus.** The virus was prepared in the same way as was the S³⁵-labeled virus except that the growth medium lacked valine instead of cystine and methionine, and uniformly C¹⁴-labeled L-valine (Radiochemical Centre, Amersham, Bucks; specific activity 5 mc/mmole) was added to 2.5 µc/ml. The specific infectivity of the purified virus was 6 × 10⁵ PFU/cpm.

**Infection of cells at 0°.** Cells were washed with PBS and resuspended in PBS containing the required amount of virus at a con-
centration of $5 \times 10^7$ cells/ml. The mixture was rocked in a siliconed tube submerged in melting ice. The rate of uptake of virus was 70 to 85% in 3 hours (50% in 1–2 hours). The suspension was then washed by diluting it fivefold into PBS at 0°, and the cells were sedimented and resuspended to $5 \times 10^7$/ml in PBS. The number of PFU adsorbed per cell was calculated from the difference between the amount of $^{32}P$ (or the number of PFU, in the case of unlabeled virus) added and that recovered in the washings after adsorption.

**Density gradient centrifugation.** Density gradients ranging from 600 to about 300 mg of potassium tartrate per milliliter (McCrea et al., 1961) were constructed in 5-ml Lusteroid tubes by collecting 2 ml of effluent from a stirred mixing chamber initially containing 2.5 ml of tartrate solution (600 mg/ml, pH 7.4); an influx of water maintained a constant volume in the mixing chamber. The gradient was usually overlaid first with 0.5 ml of virus suspended in PBS and then with 1.8 ml n-heptane. After centrifuging in a Spinco SW-39 rotor, the bottom of the tube was pierced with a needle and 3-drop fractions were collected until the flow stopped as the water-heptane interface reached the hole. Sucrose density gradients were made similarly, with sucrose solution (200 mg/ml) in the mixing chamber.

Self absorption of radioactivity in the dried fractions, although negligible with $^{32}P$, would be considerable with $^{35}S$, and would increase with the density of the fraction. However, only relative counts were required, and since the density difference between adjacent fractions was only about 5%, the changes in self-absorption would not significantly alter the apparent position of a $^{35}S$ peak.

**Measurement of radioactivity.** Samples were dried in planchette and counted to 800 counts in a Philips automatic end-window counter. Radioactivity is expressed in terms of counts per minute (cpm). The $^{35}S$ was differentially screened by placing on top of each planchette a disk of thin (card index) card (23 mg/cm²), which passed 86% of the $^{32}P$ but only 8% of the $^{35}S$ radiation.

**Acid reactivation of neutralized virus.** The phthalate buffer used by McLaren et al. (1960) was found unsatisfactory because 0.05 M phthalate at pH 2.5 rapidly inactivated virus infectivity, and the addition of 1% gelatin was not consistently protective. Glycine (0.05 M, pH 2.5), however, was innocuous without gelatin. The reactivation at pH 2.5 of debris-neutralized virus was complete within 1 minute at 20°; routinely, samples were diluted 100-fold into the acid buffer and then at least 10-fold into PBS (pH 7), or neutralized with N NaOH.

**RESULTS**

**Fate of Labeled Virus**

Joklik and Darnell (1961) reported that over 50% of the $^{32}P$ of adsorbed labeled poliovirus eluted rapidly from cells at 37°. The eluted particles sedimented like virus and were unaffected by RNase but would not adsorb to cells. We have observed a similar phenomenon but propose to call the rejection process "sloughing," since the rejected particles appear to be associated with a low density component possibly derived from the cell wall, and having some properties of a lipoprotein.

**1. Properties of the Sloughing Reaction**

After adsorption of $^{32}P$-labeled virus at 0°, 60–80% of the $^{32}P$ was sloughed from cells during 1 hour at 37° in PBS. The rest of the label remained firmly bound even after further incubation of the cells in fresh medium. At 20° the amount of $^{32}P$ sloughed during 1 hour was less than 5% of that sloughed in the same time at 37°. When the temperature was raised gradually, sloughing started at a cell temperature of 23°–24° (Fig. 1).

The sloughing of $^{32}P$ was almost completely inhibited by 10 mM glutathione (Table 1). Sloughing started again when the cells were centrifuged and resuspended in glutathione-free medium, a result indicating that the inhibition was reversible. A similar experiment with $^{14}C$-valine-labeled virus showed that the protein label was also sloughed and that glutathione inhibited this sloughing also. Oxidized glutathione was
2. Properties of the Sloughed P₃²-Labeled Material

Cells were infected (1–5 adsorbed PFU/cell) and washed at 0° as described in Materials and Methods, and then incubated for 1 hour at 37° in PBS at 5 X 10⁶ cells/ml. The supernatant, after centrifuging for 10 minutes at 500 g, contained the sloughed P₃², with a specific infectivity (PFU/cpm) between 0.1% and 1.0% of that of the original virus. This material was used for the following experiments.

a. Effect of acid on infectivity. Acidification of the sloughed material to pH 2.5 (see Process 2 below: Neutralization of infectivity by cells) caused a tenfold increase in its specific infectivity, but the PFU/cpm ratio was always less than 10% of that of the original virus.

### Table 1

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (mM)</th>
<th>P₃² sloughed in 1 hour at 37° (%)</th>
<th>Inhibition of sloughing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1</td>
<td>51</td>
<td>27</td>
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<tr>
<td>Glutathione</td>
<td>5</td>
<td>18</td>
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<tr>
<td>Glutathione</td>
<td>10</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>Oxidized glutathione⁶</td>
<td>10</td>
<td>46</td>
<td>33</td>
</tr>
</tbody>
</table>

*Cells were infected (0°, 3 hours, 4 adsorbed PFU/cell) with P₃²-labeled virus, washed, and resuspended in PBS at 0° (see Materials and Methods). At zero time they were diluted to 5 X 10⁶ cells/ml into PBS at 37° with the indicated addition. After 1 hour the suspensions were centrifuged for 5 minutes at 500 g and the sediments and supernatants were counted for cell-bound and sloughed P₃², respectively.

⁶ Containing 0.06 equivalents of SH per mole estimated by the method of Boyer (1954).
b. Adsorption. Less than 5% of the sloughed P\textsuperscript{32} adsorbed to fresh cells at 0° in 3 hours (83% of the P\textsuperscript{32} of untreated labeled virus adsorbed under the same conditions); this alone can explain the low infectivity. Further evidence that the surface of the virus had been altered by sloughing was found in the fact that the sloughed P\textsuperscript{32} adhered strongly to a column of DEAE-cellulose, from which it was eluted by 0.5 N NaOH, but not by phosphate concentrations up to 0.5 M (pH 6.7).

c. Sedimentation. To compare the sedimentation properties of the sloughed material with that of untreated virus, a mixture of S\textsuperscript{35}-labeled virus, acting as a marker, and P\textsuperscript{32}-labeled sloughed material was centrifuged through a potassium tartrate density gradient (see Materials and Methods). The two types of label were readily distinguishable by differential screening (see Materials and Methods).

Figure 2A shows that, after 2 hours' centrifugation at 30,000 rpm the original (untreated) P\textsuperscript{32} virus was in the same position as the marker S\textsuperscript{35} virus, but the sloughed P\textsuperscript{32} (Fig. 2B) formed a distinct band at a lower density. After 4 hours' centrifugation at 30,000 rpm (Fig. 2C), the marker virus had reached the bottom of the tube, but the sloughed P\textsuperscript{32} had moved very little and so was near its equilibrium position. In tartrate gradients set up for calibration purposes, the density of the fractions equivalent to the equilibrium position of the sloughed P\textsuperscript{32} was found to be 1.20-1.22 g/ml.

Joklik and Darnell (1961) reported that their eluted material sedimented at the same speed as virus in a sucrose density gradient. However, by including the S\textsuperscript{35}-labeled virus as a marker (which eliminated the effect of slight variations between individual gradients) it was found (Fig. 3) that the sloughed P\textsuperscript{32} sedimented slightly more slowly than virus in a sucrose density gradient. At the relatively low densities of the sucrose gradient, the separation was less efficient than in tartrate gradients, which can be used at densities high enough to establish equilibrium.

The low density of the sloughed virus showed that the particles had either lost all or most of their RNA or acquired a component of low density, such as a lipoprotein. The first alternative is unlikely as all the sloughed P\textsuperscript{32} sedimented and appeared in a single band in the density gradients, giving no evidence of free or degraded RNA.
d. Sensitivity to trypsin. An attempt was made to detach the supposed light component. The sloughed P\textsuperscript{32}-labeled material was incubated with trypsin as described in Fig. 4, and then centrifuged through a tartrate density gradient. The usual peak of sloughed P\textsuperscript{32}, seen in the control gradient (Fig. 4A), disappeared after trypsin treatment and was replaced by two smaller peaks, one in the position characteristic of virus and one at the top of the tube, above the tartrate gradient (Fig. 4B); the top peak may indicate some breakdown of labeled material into a nonsedimentable form. The reappearance of particles that sedimented like virus suggests that the low density of the original sloughed particles was due to the attachment of light material rather than to an increased degree of hydration. Trypsin treatment did not, however, restore the infectivity of the sloughed material.

e. Effect of ether. A mixture of sloughed P\textsuperscript{32}-labeled virus and untreated S\textsuperscript{35}-labeled virus was shaken by hand at frequent intervals, with an equal volume of ether, for 1 hour at 20°. After centrifuging for 10 minutes at 500 g, 90% of the P\textsuperscript{32} but only 14% of the S\textsuperscript{35} had been removed from the aqueous phase. The missing radioactivity did not appear in the ether layer and was presumably trapped in the precipitate at the interface. Such ether treatment has no effect on the infectivity of poliovirus.

3. Fate of Cell-Bound P\textsuperscript{32}

The P\textsuperscript{32} of virus which remained attached to the cell after sloughing was examined to see whether it was changed to a form that was susceptible to RNase. Cells infected at 0° (as described in Materials and Methods) with P\textsuperscript{32}-labeled virus (3 adsorbed PFU/cell) were incubated for various times at 37° in medium CSV6 and then disrupted by five cycles of freezing (at −70°) and thawing (at 37°). The debris was incubated for a further 2 hours at 37° with 10 μg RNase per milliliter. The amount of breakdown of labeled material was calculated as the proportion of the P\textsuperscript{32} which remained in the supernatant after (a) centrifuging the RNase-treated mixture for 90 minutes at

![Figure 3. Sedimentation of untreated and sloughed poliovirus in a sucrose density gradient (200–100 mg/ml). A mixture of untreated S\textsuperscript{35}-labeled virus and sloughed P\textsuperscript{32} was centrifuged through a sucrose density gradient (see Materials and Methods) for 30 minutes at 30,000 rpm. The radioactivity of 2-drop fractions was measured with and without card screens, and the activities of P\textsuperscript{32} (△) and S\textsuperscript{35} (○) were calculated.](image-url)
Relation of Fate of $\text{P}^{32}$ to Fate of Infectivity

The fate of the infectivity of adsorbed poliovirus was next examined, in an attempt to assess the significance of the observed fates of viral $\text{P}^{32}$ in relation to the infection of the cell.

A potentially infective particle may undergo four separate early processes which may be defined and distinguished as follows.

**Process 1:** Adsorption to cells occurs at 0°, but, as shown in Fig. 5, full infectivity was recovered on disrupting the cells with deoxycholate (DOC). A somewhat higher infectivity was in fact obtained by DOC treatment of cell-adsorbed virus than of the original free virus, which was unaffected by

100,000 g or (b) adding an equal volume of N HCl in 76% ethanol at 4° and removing the precipitate by light centrifugation. Both methods gave the same result: by 2 hours after infection, only 14% of the cell-bound $\text{P}^{32}$ was susceptible to RNase as compared with 12% at 1 hour and 7% at 1 minute. Thus only a few of the nonsloughed particles reached a RNase-sensitive stage, and these did so within an hour. Whether or not these were the infecting particles is uncertain.

**Fig. 4.** Effect of trypsin on the density gradient pattern of sloughed $\text{P}^{32}$. A mixture of untreated $\text{S}^{35}$-labeled virus with (A) sloughed $\text{P}^{32}$, or (B) sloughed $\text{P}^{32}$ treated with crude trypsin (Difco 1:250, 2.5 mg/ml for 2 hours at 20°), was centrifuged for 2 hours at 35,000 rpm through a tartrate gradient (see Materials and Methods). The radioactivity of 3-drop fractions was measured with and without card screens, and the activities of $\text{P}^{32}$ (A) and $\text{S}^{35}$ (O) were calculated.

**Fig. 5.** Distinction between adsorption and neutralization of infectivity by cells. At zero time, a suspension of $5 \times 10^7$ cells/ml in PBS was mixed, at 0°, with virus (filled symbols), and rocked in melting ice in a silicone-treated tube; a control without cells was included (open symbols). At intervals, replicate samples were taken and abruptly diluted 50-fold into either (1) DOC (2 mg/ml in PBS), and assayed for total unneutralized infectivity (triangles), or (2) GCM, and at once centrifuged for 5 minutes at 500 g. DOC (2 mg/ml) was added to the cell-free supernatants from samples (2) and they were assayed for unadsorbed infectivity (circles).
There was no loss or gain of infectivity in the absence of cells.

Process 2: Neutralization of infectivity by cells involves the formation of a more stable cell-virus link with loss of infectivity; infectivity is not restored by DOC. This link, like the virus-antibody bond (Mandel, 1961) is acid labile, and viral infectivity is restored at pH 2.5. A similar acid-reversible neutralization of infectivity has been reported when disrupted HeLa cells were incubated with poliovirus at 37° (Holland and McLaren, 1959; McLaren et al., 1960), and this we have confirmed using ERK cell homogenates. Neutralization by cells has earlier been described as “eclipse,” meaning simply loss of infective centers on disruption of cells (Darnell, 1958; McLaren et al., 1959), but this term now seems undesirable in view of the reversible nature of the inactivation.

Process 3: Development of antiserum resistance results in the ability of an infected cell to form an infective center despite the brief application of potent antiviral serum. It implies that an infecting particle has either penetrated the cell wall or has in some other way become inaccessible to antibody.

Process 4: Acid-irreversible eclipse—the proportion of the adsorbed infectivity which is lost and cannot be recovered by acid treatment is here described as having undergone acid-irreversible eclipse. This term may include a number of processes, either reversible or irreversible, but no means of recovering the infectivity have yet been found.

Purified poliovirus preparations contain many more viruslike particles than detectable PFU (Schwerdt and Fogh, 1957). It must therefore be borne in mind that whereas the measurements of P₃₂ already described are related to all the adsorbing labeled particles, measurements of infectivity (in the experiments to be described below) are concerned only with those particles which are potentially infective, while measurements of infective centers can involve only that fraction of the potentially infective particles which actually causes infection.

1. Sloughed P₃₂

It has been observed that at 20°, or in the presence of 10 mM glutathione at 37°, sloughing was prevented. It was therefore of interest to know, in considering the significance of sloughing in relation to infection, to what extent other processes (viz. those defined above) occurring soon after adsorption were also prevented.

In the experiment described in Table 2, cells with P₃₂-labeled virus adsorbed at 0° were incubated at 20°. Samples were taken at intervals and assayed, to determine (a) the amount of the adsorbed infectivity neutralized by the cells, (b) the amount recoverable by acid treatment, (c) the proportion of infective centers that had developed resistance to antiserum, and (d) the proportion of the P₃₂-labeled particles that had been sloughed. It was found that the infectivity was rapidly neutralized by the cells at 20° and that antiserum resistance also developed, although rather more slowly. But very little acid-irreversible eclipse occurred, and virtually no sloughing. The cell suspension was then warmed to 37°, whereupon most of the infectivity quickly became irreversibly eclipsed, and 80% of the adsorbed P₃₂ was sloughed (Table 2).

Three conclusions may be drawn from this experiment. First, antiserum resistance developed (at 20°) without sloughing, which appears to rule out the possibility that infection involves a process analogous to sloughing but directed (with a probability of 20–40%) into the cell. Secondly, sloughing occurred to the normal extent (at 37°) despite the fact that, at 20°, 91% of the infectivity had been neutralized by the cells and 58% of the infective centers had become resistant to antiserum. Therefore the process preceding sloughing did not compete directly for infective particles with the process of neutralization of infectivity by the cell, nor with the development of antiserum resistance. Thirdly, sloughing and acid-irreversible eclipse were not clearly distinguishable by their rates at 20°.

Another experiment was carried out to confirm that particles which would normally have been sloughed could not be diverted to infectious pathways by a preincubation at
20° (during which sloughing was prevented but development of antiserum resistance proceeded). A cell suspension infected at 0° (0.01 adsorbed PFU/cell) was divided into 2 parts which were incubated for up to 3 hours in GCM at 20° and 37°, respectively; during this time they were assayed for infective center content. The number of infective centers produced at 20° was 0.007–0.009 per cell, no greater than that produced at 37° (0.010–0.012 infective centers per cell). The failure to demonstrate competition in these experiments may indicate either (a) that the fate of the particles is decided at the time of adsorption or soon after: a proportion is neutralized by the cell in a pre-sloughing state and cannot subsequently cause infection, while the rest become permanently attached to the cell, or (b) the presence of a distinct component of the virus preparation which always tends to be sloughed (and therefore has a low specific infectivity), or (c) that the competition, if it exists, occurs at a stage later than the development of antiserum resistance.

In a further experiment, described in Table 3, cells were infected at 0° and diluted into medium at 37° containing glutathione sufficient to cause a substantial inhibition of sloughing. Infectivity assays during incubation at 37° showed that neutralization of the adsorbed infectivity by the cells was not affected by glutathione, but that in the presence of glutathione much more of the infectivity remained recoverable at pH 2.5 than in its absence. Thus, as was the case on incubation at 20° (Table 2), glutathione treatment also clearly distinguished sloughing from the process of neutralization by cells, but not from acid-irreversible eclipse.

2. Cell-Bound P32

Having observed that the low specific infectivity (PFU/cpm) of the sloughed P32-
labeled material could not be restored at pH 2.5, it was of interest to see whether the nonsloughed material was irreversibly eclipsed to the same extent. Figure 6 shows that this was not the case. One hour after infection, when sloughing had virtually ceased, the specific infectivity of the cell-bound labeled material was restored to about 60% of that of the original virus by acid treatment. At this time 90% of the originally adsorbed infectivity was not recoverable by acid treatment. Like the lack of competition between the processes of sloughing and infection, this result may be interpreted in one of two ways, again depending on whether or not the sloughed particles were originally potentially infec-

**TABLE 3**

<table>
<thead>
<tr>
<th>Minutes at 37°</th>
<th>% Neutralized by cells</th>
<th>% Nonrecoverable at pH 2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No glutathione</td>
<td>5 mM glutathione</td>
</tr>
<tr>
<td>40</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td>80</td>
<td>89</td>
<td>88</td>
</tr>
</tbody>
</table>

* Cells were infected (0°, 30 minutes, 4 adsorbed PFU/cell) with unlabeled virus, washed, and resuspended in PBS at 0° (see Materials and Methods). A sample was diluted into DOC (2 mg/ml in PBS) for assay of adsorbed unneutralized infectivity. At zero time the infected cells were diluted to 10^6 cells/ml into GCM at 37° with or without 5 mM glutathione. Replicate samples were taken at intervals and assayed after (i) dilution through DOC solution or (ii) freezing to −70° five times and dilution through pH 2.5 buffer followed by neutral buffer.

* Expressed as

\[
\left(1 - \frac{\text{infectivity recovered in samples (i)}}{\text{infectivity in initial DOC lysate at 0°}}\right) \times 100.
\]

* Expressed as

\[
\left(1 - \frac{\text{infectivity recovered in samples (ii)}}{\text{infectivity in initial DOC lysate at 0°}}\right) \times 100.
\]

**DISCUSSION**

The adsorption of poliovirus to ERK cells at 0° results in a weak linkage which is broken by DOC with full recovery of infectivity. When the temperature is raised, most of the infective particles rapidly be-
come more firmly bound, a process resulting in neutralization of infectivity by the cells. The cell-neutralized particles are still intact, since nearly all the infectivity is regained at pH 2.5 if subsequent processes are prevented by incubation at 20°. In this respect they resemble particles neutralized by cell debris (McLaren et al., 1960), and by antibody (Mandel, 1961). The rate of neutralization by cells is considerably greater than the rate at which those particles which will initiate infection become resistant to antiserum. This implies that neutralization of infectivity by the cell takes place outside the cell, assuming that antibody does not affect intracellular virus (Howes and Melnick, 1957).

At temperatures above 23°, between 60% and 80% of the P32 from adsorbed labeled particles is sloughed from the cell, apparently as intact particles in association with a component of low density, possibly lipoprotein derived from the cell wall. The sloughing process is reversibly inhibited by glutathione, but not by other reducing agents. The sloughed P32 will not reabsorb to fresh cells and is not reactivated at pH 2.5.

Particle-to-PFU Ratio

A high ratio of particles to PFU may reflect either a high proportion of noninfective virusslike particles or a small chance that each particle will achieve infection even though all are potentially infective (Taylor and Graham, 1961). Taylor and Graham (1961) found about 50 electron-microscopically visible particles for each PFU in Dowex-purified virus. Joklik and Darnell (1961) estimated a ratio of 270 from the optical density of virus purified with DEAE cellulose, and a similar calculation from optical density gives a ratio of between 500 and 1000 for the virus used here. This difference may be related to the fact that Joklik and Darnell, using a different cell strain, observed a much higher proportion of RNase-sensitive labeled material than we did in the cell-bound fraction.

Sloughing accounts for a factor of 3 or 4 in the particle-to-PFU ratio, but it cannot yet be decided whether or not the sloughed material is derived from potentially infective particles. Joklik and Darnell (1961) concluded that the sloughed ("eluted") particles were originally infective because they had adsorbed to the cell in the first place and still contained infective RNA. This is not conclusive evidence, however, since heat- or antiserum-treated virus can have similar properties and yet prove to be noninfective (Cooper, unpublished data). The only additional evidence presented here is that glutathione inhibited both sloughing of P32 and the acid-irreversible eclipse of infectivity. This shows that the sloughed particles resemble infective particles at least in that one stage in each of their respective pathways is blocked by glutathione.

If the sloughed particles were originally infective, they have followed an abortive pathway (i.e., one not leading to infection of the cell). Furthermore, much of the infectivity of the nonsloughed virus was still recoverable at pH 2.5 one hour after infection, suggesting the presence of other unproductive adsorption sites. If, on the other hand, the sloughed particles were not potentially infective, then the overall acid-irreversible eclipse (a loss of about 90% of the adsorbed infectivity, one hour after infection) concerns only the nonsloughed particles, and there is no reason to postulate abortive pathways.

**Difficulties of Interpretation**

It has been pointed out above that a high particle-to-PFU ratio complicates comparisons between measurements of virus P32, virus infectivity, and infective centers. In fact, direct evidence for the fate of the small minority of particles which actually initiate infection is still lacking. For instance, the process of neutralization of infectivity by the cells is undergone by most (90–99%) of the adsorbed infective particles, but it is not proved to be a necessary intermediate stage in infection unless less than one potentially infective particle (as distinct from one PFU) per infective center remains unneutralized. Otherwise the possibility persists that one unneutralized particle might enter the cell and cause infection. In order to
demonstrate the essential nature of the process for infection, more than \((n - 1)/n\) of the adsorbed infectivity must undergo neutralization by the cell, where \(n\) is the number of potentially infective particles per PFU in the virus preparation. This has so far not been shown for poliovirus, and so the concept of eclipse, in the sense of loss of infectivity of the infecting particle, exists more by virtue of presumption than of proof.

In a very recent paper, Holland (1962) has suggested that the acid-irreversible eclipse is the same process as the development of antiserum resistance. Our results do not confirm this since, at 20°, the rate of development of antiserum resistance was considerably greater than the rate of acid-irreversible eclipse. This, however, does not eliminate the possibility that the small proportion of infecting particles become nonrecoverable by acid at the same time as they become resistant to antiserum.

ACKNOWLEDGMENT

We are grateful to Mr. D. Pelling for his thoughtfully competent assistance.

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Indirect Evidence for the Presence of Ribonucleic Acid in Vesicular Stomatitis Virions

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Concentrations of 5 µg/ml or less of 5-fluorodeoxyuridine (FDU) and of 5-bromo-deoxyuridine (BDU) inhibited the growth of adenovirus type 5 and of vaccinia virus in ERK and chick embryo cells, provided that adequate time was allowed for the inhibitors to act. With the same proviso, FDU and BDU also inhibited the growth of ERK cells. Under similar conditions of cultivation, concentrations of up to 100 µg/ml of FDU and of BDU had no effect on the growth of either the New Jersey or the Indiana serotype of vesicular stomatitis virus (VSV) in either ERK or chick embryo cells. It is concluded that virus particles of both serotypes of VSV contain RNA rather than DNA.

INTRODUCTION

It is difficult at present to relate the two serotypes of vesicular stomatitis virus (VSV) to other viruses. These serotypes are themselves antigenically unrelated to one another, although biologically similar. There is evidence that arthropod vectors may be involved in the natural spread of vesicular stomatitis virus, and Mussey and Suarez (1962) have demonstrated directly that VSV can grow in Aedes aegypti mosquitoes. But despite its convenience, the use of habitat as a primary criterion for classification of animal viruses presents a number of anomalies (Cooper, 1961a). As an example of such an anomaly, the structure of VSV particles (Reczko, 1960; Howatson and Whitmore, 1962) does not resemble that of other arboviruses, and in fact may be unusual or even unique among the smaller viruses that infect vertebrates.

It has been suggested (Cooper, 1961a) that for certain purposes there is merit in a formal classification of animal viruses based on the type of nucleic acid contained in the virus particle as a primary criterion. The work reported here was done with this in mind and uses the suggestion of Salzman (1960) that inhibitors of DNA synthesis can be used to differentiate between viruses containing RNA and those containing DNA.

This paper demonstrates the conditions under which two inhibitors of DNA synthesis, 5-fluorodeoxyuridine (FDU) and 5-bromo-deoxyuridine (BDU), were effective in the virus growth systems employed. Using these conditions, it is shown that FDU and BDU have no effect on the growth of either serotype of VSV.

MATERIALS AND METHODS

Samples of FDU and BDU were obtained through the courtesy of Dr. Ronald Ross, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland.

Vesicular stomatitis virus, obtained from the Research Institute, Pirbright, Surrey, England, was grown and assayed in 16-hour chick embryo cell monolayers. The plaque method described by Cooper (1957a) was used, with the difference that the liquid medium was CSV.6 (Cooper et al., 1959), and the agar overlay medium was that de-
scribed by Cooper (1961b). Titres are expressed as plaque-forming units per millilitre (PFU/ml).

Poliovirus was assayed by the agar cell-suspension plaque method (Cooper, 1961b), in ERK cells grown as described by Cooper et al. (1959). Titres are expressed as PFU/ml.

Adenovirus type 5, obtained from the National Institute for Medical Research, Mill Hill, London, was grown in ERK cells in CSV.6, and assayed in the same cells by the following end-dilution method. Serial tenfold dilutions of a preparation of unknown titre were added in 0.1-ml amounts to 16-hour tube cultures (8 tubes per dilution), each tube containing $2.4 \times 10^4$ ERK cells in 2 ml CSV.6. Cytopathic effect (CPE) was recorded after 8 days at 36°; the dilution factor which produced detectable CPE in half of the tubes at this time was interpolated, and the titres (which equal the reciprocal of the dilution factor) are expressed as tissue culture infectious doses per millilitre.

Vaccinia virus was obtained both from the Lister Institute, Elstree, Hertfordshire, England, and from the Pasteur Institute, Iran. Its infectivity was assayed as for adenovirus except that CPE was recorded after 4 days at 36°.

RESULTS

Effect of FDU on Growth of Adenovirus Type 5 in ERK Cells and of Vaccinia Virus in ERK and Chick Embryo Cells

The efficacy of FDU in inhibiting DNA synthesis in the systems to be used to test VSV was first checked by its effect on the growth of two viruses known to contain DNA (vaccinia virus, Wyatt and Cohen, 1953; adenovirus type 5, Allison and Burke, 1962). Table 1 shows that, when the inoculum was very small, FDU inhibited the CPE of adenovirus in ERK cells, and of vaccinia virus in ERK and chick embryo cells. The inhibition was complete down to 3 μg/ml, and partial at some lower concentrations. Production of infectivity was strongly inhibited by 3 μg FDU/ml. However, FDU failed to protect ERK cells against somewhat larger inocula, a result suggesting that in these cases FDU had not been present sufficiently in advance of the final cycle of virus growth.

Effect of FDU on the Growth of ERK Cells

The experiments described in the preceding paragraph show that FDU was not always able to inhibit DNA synthesis in the cell system used. In order to be sure that FDU was exerting its full effect, ERK cells were henceforth incubated with FDU before infection until the cells had ceased to divide; it is presumed that DNA synthesis will then also have ceased. The following experiment indicated the amount of preincubation with FDU required to stop ERK cell division.

Twelve replicate ERK cell cultures were set up in Pyrex bottles; 6 of the cultures contained 10 μg of FDU per millilitre. The cultures were incubated at 36° without change of medium at any stage, and at intervals one control and one FDU-containing culture were trypsinized and the cells were counted. Figure 1 shows the total recoveries of cells in both sets of cultures; the inhibition of the growth of ERK cells was not complete until 4–5 days after the addition of FDU. A similar experiment with 50 μg/ml of FDU indicated that inhibition by this higher concentration was not complete until 3 days after the addition of FDU.

Effect of FDU on the Growth of VSV and Poliovirus in ERK and Chick Embryo Cells

Cells in which the growth of VSV was to be tested were preincubated with concentrations of FDU (50 and 100 μg/ml) and for a time (7 days) in excess of time and concentration shown to be sufficient for inhibition of the growth of ERK cells (see Fig. 1) and of DNA-containing viruses (see Table 1). Poliovirus was included in the test series as a virus known to contain RNA (Schwerdt and Schaffer, 1955), and was treated in the same way as was VSV. The results of the tests of virus growth were measured in terms of production of infectivity and of CPE. However, since several cycles of virus growth must occur if the presence of CPE is to indicate synthesis of viral nucleic acid, very small inocula of VSV and poliovirus were used. This additionally provided a further period of up to 24 hours in the presence
Table 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell type</th>
<th>Inoculum (infectious doses per tube)</th>
<th>FDU (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Adeno</td>
<td>ERK</td>
<td>1000</td>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td></td>
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<td>3</td>
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<td></td>
<td></td>
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<td></td>
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<td>0</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>ERK</td>
<td>100</td>
<td>3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>3</td>
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<td></td>
<td></td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>Chick embryo</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10&lt;sup&gt;e&lt;/sup&gt; to 10</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> One series of tubes received 2.4 × 10<sup>6</sup> ERK cells in 2 ml CSV.6, and another series received 5 × 10<sup>6</sup> chick embryo cells in 1 ml CSV.6. FDU was added to the indicated concentrations, and the tubes were incubated at 36°. After 5 hours, 0.1 ml of CSV.6 containing the indicated inocula were added to the tubes. Incubation was continued, and the tubes were examined daily for CPE. Control cells in absence of virus were morphologically normal at the end of the experiment, although those in presence of 10 and 30 μg/ml of FDU were slightly changed. Relative CPE is expressed as 0, 1, 2, or 3, indicating, respectively, zero, up to 10%, 10-90%, or all cells showing CPE. All tubes were duplicated, and both readings of CPE are given if duplicates differed.

<sup>b</sup> Relative CPE after 8 days at 36°.
<sup>c</sup> Infective titre from an end-dilution method, of material harvested at the time for which CPE is reported.
<sup>d</sup> Relative CPE after 4 days at 36°.
<sup>e</sup> Relative CPE after 7 days at 36°.

of FDU before the final cycle of virus growth occurred.

Table 2 indicates that the infectivity produced by either serotype of VSV in chick embryo or ERK cells was independent of the presence of FDU. Poliovirus growth was also unaffected.

CPE was complete in all infected cultures, and there was no significant difference in the rate of appearance of CPE in presence or absence of FDU in any particular cell-virus combination of the experiment described in Table 2. The size of the inoculum, within the limits used, also had no detectable effect on production of infectivity or of CPE. Uninfected control cells showed no cytopathic effect resembling that of virus, although FDU had produced slight morphological changes in the cells by the end of the experiment.

**Effect of BDU on Growth of VSV in Chick Embryo and ERK Cells**

As a control of the efficacy of BDU in chick embryo cells, two 1-liter flasks each containing a monolayer of 10<sup>6</sup> chick embryo cells were infected with about 200 tissue cultures infective doses of vaccinia virus in 2 ml CSV.6. After 1 hour's adsorption, the cultures received 100 ml of medium CSV.6, and BDU was added to one culture to a concentration of 5 μg/ml. Incubation proceeded for 4 days at 36°, when the culture without BDU contained fifty to one hundred macroscopically visible plaquelike areas of morphologically altered cells, which became
confluent by 6 days. The culture in presence of BDU remained morphologically unchanged. Infectivity tests were not performed.

In order to test the effect of BDU on the growth of VSV in chick embryo cells, three series of tubes containing $3 \times 10^5$ chick embryo cells in 1 ml of medium CSV.6 were incubated for 7 days in absence of BDU or in presence of 50 or 100 $\mu$g BDU/ml, respectively. Tubes of each series were then infected by the addition of 0.1 ml medium containing either (a) 1, 10, or 100 PFU of the Indiana serotype of VSV, or (b) 1, 10, or 100 PFU of the New Jersey serotype of VSV, or (c) no virus; each tube was duplicated.

Whether in presence or absence of BDU, complete viral cytopathic effect was present in all infected cultures after a further 2 days at 36°; at this time cultures were harvested and duplicate tubes pooled for assay of infectivity. Uninfected control cells showed no cytopathic effect resembling that of virus, although BDU had produced slight morphological changes in the cells by the end of the experiment.

No marked effect of BDU on the production of VSV infectivity was found. Control

### Table 2

**The Effect of FDU on the Growth of the Indiana and New Jersey Serotypes of VSV and Poliovirus in ERK$^a$ and Chick Embryo$^b$ Cells**

<table>
<thead>
<tr>
<th>Virus inoculum$^c$</th>
<th>Cell type</th>
<th>FDU concentration, $\mu$g/ml</th>
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<tr>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>ERK</td>
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</tr>
<tr>
<td>VSV (New Jersey)</td>
<td>Chick embryo</td>
<td>10 to 200 $\times 10^4$</td>
</tr>
<tr>
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<td>ERK</td>
<td>10$^4$ to 10$^5$</td>
</tr>
<tr>
<td>VSV (Indiana)</td>
<td>Chick embryo</td>
<td>15 to 35 $\times 10^6$</td>
</tr>
<tr>
<td>VSV (Indiana)</td>
<td>ERK</td>
<td>10$^4$ to 10$^6$</td>
</tr>
</tbody>
</table>

$^a$ Two series of tubes containing $8 \times 10^6$ ERK cells in 1 ml of CSV.6 were incubated at 36° for 7 days in presence of 50 or 100 $\mu$g FDU per milliliter, respectively; an identical control series containing no FDU was set up on the sixth day.

$^b$ Three series of tubes each containing $3 \times 10^8$ chick embryo cells in 2 ml CSV.6 were incubated at 36° for 7 days in presence of zero, 50, or 100 $\mu$g FDU per milliliter, respectively.

$^c$ On the seventh day, all tubes received 0.1 ml of CSV.6 containing either zero, 1, 10, 100, or 1000 PFU. All samples were duplicated. Tubes were examined daily for CPE, and harvested 3 days (poliovirus in ERK cells, and VSV in chick embryo cells) or 3 to 8 days (VSV in ERK cells) after infection, when viral CPE was complete in all infected cultures. Duplicate tubes were pooled for assay of infectivity.

$^d$ Values are range of yields produced, in PFU/ml.
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Fig. 2. Effect of BDU on the growth of ERK cells. Two series of 12 cultures, each containing 5 x 10^5 ERK cells in 5 ml CSV.6, were placed in Pyrex bottles at 36° at zero time. One series contained 100 µg BDU/ml. One culture from each series was trypsinized at the times indicated on the abscissa, and the cells were counted. Less than 5% of the inoculum and of all harvested cells absorbed stain from trypan blue solution (2 mg/ml). After cell growth had ceased in the cultures containing BDU, these cultures were used for the growth of both serotypes of VSV (see text).

cultures infected with the Indiana serotype produced 2 to 5.5 x 10^6 PFU/ml, whereas 50 and 100 µg BDU/ml, respectively, permitted production of 1.5 to 2.75 x 10^6 and 0.65 to 1.6 x 10^6 PFU/ml of this virus. All cultures of the New Jersey serotype produced between 0.84 and 2.5 x 10^5 PFU/ml, whether in presence or absence of BDU.

In order to test the effect of BDU on the growth of VSV in ERK cells, cultures of ERK cells were preincubated in presence of 100 µg BDU/ml until DNA synthesis could safely be presumed to be inhibited. The growth of these cultures is compared in Fig. 2 with the growth of cultures containing no BDU. Growth of ERK cells had ceased in presence of BDU by the sixth day, but incubation was continued until the tenth day, when the BDU-containing cultures were infected in duplicate with about 500 PFU of either the New Jersey or the Indiana serotype of VSV; two cultures were left uninfected. As controls, fresh cultures had been set up in the absence of BDU on the seventh day, and were infected in the same manner and at the same time as the BDU-containing cultures. The number of cells per culture was about the same in control and in infected cultures. Incubation continued at 36° for 4 days after infection, by which time appreciable degeneration was evident in the noninfected cultures containing BDU. CPE was then complete in all cultures infected with the Indiana serotype, and partial in all cultures infected with the New Jersey serotype. The extent and rate of onset of CPE were identical in cultures with or without BDU. On the fourth day after infection, the cultures were harvested and duplicates were pooled for assay of infectivity. Preincubation in presence of BDU produced no significant effect on the yields of infectivity: the Indiana serotype yielded 1.3 x 10^6 PFU/ml in absence of and 1.0 x 10^6 PFU/ml in presence of BDU, and the New Jersey serotype yielded 10^4 PFU/ml in absence and 7.5 x 10^3 PFU/ml in presence of BDU.

DISCUSSION

The chemical structure of FDU and BDU lead to the expectation that both analogs will interfere with DNA synthesis, and this expectation is borne out in practice (Cohen et al., 1958; Simon, 1961; Wilson and Dinning, 1961). Therefore growth of DNA-containing viruses should also be inhibited by these analogs, and this also is substantiated (Salzman, 1960; Simon, 1961). The effect on growth of RNA-containing viruses is less easy to predict, but Salzman (1960) has reported that FDU has no effect on the growth of poliovirus whereas vaccinia virus is markedly inhibited. Simon (1961) has used the lack of effect of amionopterin, 5-fluorouracil, and BDU on the growth of known RNA-containing viruses (poliovirus and Newcastle disease virus) to conclude that synthesis of viral RNA is, in these instances at least, independent of cellular DNA or DNA synthesis. Thus, provided that the analogs can be shown to be effective in the system used for growing the virus, there seems to be good reason for accepting the lack of effect of FDU and
BDU on the growth of a particular virus as indicating that the infective particles formed contain no DNA, and therefore presumably contain RNA. However, in seeking to demonstrate such a negative result, it is desirable to use doses considerably in excess of the smallest effective dose. It is also desirable to use both FDU and BDU since they probably act on different stages of the infectivity and before cell breakdown became apparent; these particles sedimented in the ultracentrifuge and adsorbed to infusorial earth in a fashion similar to the infectivity.

ACKNOWLEDGMENTS

We are grateful to Dr. Ronald B. Ross for generous gifts of FDU and BDU, and to Miss Jennifer Constable for skilful and extensive technical assistance.

REFERENCES


Aspects of the Growth of Poliovirus as Revealed by the Photodynamic Effects of Neutral Red and Acridine Orange

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The sensitivity to visible light of poliovirus, grown in the presence of neutral red (NR) or acridine orange, increases with concentration of the dyes. Simple mixing of dye and mature virus, whether it occurs inside or outside the cell, has no effect. At a concentration of NR (4 μg/ml) at which virus and cell growth are little affected in darkness, light sensitivity is found to be acquired at or just before the onset of virus maturation, and no earlier portion of the growth cycle is even transiently light sensitive. The presence of NR is not required much before the onset of maturation in order to render nearly all the virus progeny light sensitive. Provided that virus maturation has not already ceased, irradiation with light of intensity sufficient to inactivate nearly all the mature NR virus in a virus-cell complex nevertheless permits some further maturation to occur. However, the amount of virus formed after light treatment is less than that which would have matured in an equivalent time in unirradiated infected cultures.

NR virus rapidly loses its light sensitivity after adsorption to fresh cells. This loss is not due to simple entry of an intact NR virus particle into the cell, as NR virus (produced and retained intracellularly) is fully light sensitive. The rate of loss of light sensitivity after adsorption is similar in presence or absence of NR. This rate is also very similar to the rate of loss of light sensitivity of virus grown in acridine orange, and to the rate of gain of antiserum resistance of the infective center. When light-sensitive virus is used to infect cultures in the absence of NR, the virus progeny is completely light resistant.

INTRODUCTION

In the presence of neutral red (NR), poliovirus becomes sensitive to light at or about the time of maturation during virus growth (Wilson and Cooper, 1962). Such light-sensitive virus loses its sensitivity to light soon after adsorption to fresh cells, whether in the continued presence of NR or, as described below, in its absence. This paper presents further aspects of the photodynamic effects of NR. In addition, some experiments are presented on the photodynamic behaviour of virus grown in the presence of acridine orange.

MATERIALS AND METHODS

Cells and virus. ERK cells were grown in medium CSV.6 as described by Cooper et al. (1959). The virus used was poliovirus type 1 (Brunenders) and was grown and assayed in ERK cells by the agar cell-suspension plaque method (Cooper, 1961). Assay plates were incubated in a completely darkened box, and the results are expressed as plaque-forming units per millilitre (PFU/ml). "Normal" virus was grown in absence of NR, and virus grown in a standard way in
presence of 4 µg NR/ml is referred to as "NR virus"; all virus used as an inoculum for experiments had been clarified by centrifugation for 15 minutes at 10,000 rpm in the Spinco 40 rotor, sedimented for 90 minutes at 40,000 rpm, and resuspended in PBS to one-tenth of the original volume.

Source of dyes. NR was supplied by Hartman-Leddon Co., Philadelphia, Pennsylvania; the colour index was number 825, and the total dye content was given as 92%. Michrome acridine orange (FS) was supplied by E. Gurr Ltd., London, England.

Safe-working light. All manipulations of cultures or virus grown in NR were carried out rapidly in a completely darkened room under indirect red lighting which was as dim as practicable. The light sources were either a 30-watt tungsten filament lamp with a 20 µg/ml aqueous NR solution as filter, or a 15-watt red bulb. These sources were suspended 60-70 cm above the bench surface. Exposure for 20 minutes at bench level to the direct beam resulted in no detectable inactivation of a preparation of NR virus.

Experiments involving monolayers. Most experiments employed replicate 17-hour monolayers of ERK cells, formed by adding 10^6 cells in 5 ml of medium CSV.6 to Pyrex bottles and incubating them overnight at 35°; the culture area was 15 cm^2. The concentration and time of addition of NR or acridine orange are indicated in the text.

Virus infection of monolayers was as follows, all operations being performed in a room at 35°. The medium was removed and kept; 3 ml of phosphate-buffered saline (PBS) containing 2-3 × 10^5 PFU were added and left for ½ hour; the cultures were then drained and washed twice with 5 ml PBS, and the original medium was returned to the culture bottles. Under these conditions, almost all cells showed a cytopathic effect typical of virus action after 6 hours, and further increase of infectivity did not occur after 7-8 hours, so that all cells were infected initially. Virus yields were harvested at the times indicated in the text by the addition to the whole cultures of sodium deoxycholate to 2 mg/ml, and the lysates were stored if necessary at −20° in darkness for subsequent assay of infectivity.

A few experiments employed confluent monolayers contained in 6-cm petri dishes. They were formed by the same procedure as for bottle cultures except that the cultures contained 5 × 10^6 cells in 5 ml CSV.6, and were incubated in an atmosphere of 5% CO₂ in air. The methods for infection of these cultures are described in the text.

Conditions used for photoinactivation. The conditions for irradiation were such that a maximum light intensity was delivered to the sample without a resulting rise in temperature in that sample. Cultures were kept at 35 ± 0.1° C during irradiation and in darkness. Unless otherwise stated, material to be irradiated was contained in colourless Pyrex bottles or petri dishes in a layer <2 mm deep. When it was necessary to irradiate samples which had been previously incubated in different concentrations of the dye, the samples were either diluted to the same concentration of NR to standardize its light filtering effect or else diluted until almost no colour was perceptible (to 0.4 µg NR/ml).

Containers to be irradiated were placed 46 cm below an 80-watt "daylight" fluorescent strip 147 cm in length, in a room kept at 35°; under these conditions NR virus was 90% inactivated within 3 minutes. The light intensity, as measured by the electrical resistance of a cadmium sulphide photodiode cell, varied by less than 5% for replicate samples. Except for manipulations under the safe light, and during periods of light treatment, cells and virus containing NR or acridine orange were maintained in complete darkness.

**Results**

**The Lack of Effect of Light and Neutral Red or Acridine Orange on the Infectivity of Mature Poliovirus**

A virus preparation containing 15 × 10^7 PFU/ml of normal virus was incubated for 8 hours in PBS, pH 7.1 at 35°, (A) in the absence of NR, and (B) in the presence of NR (40 µg/ml). After incubation, the titres were: (A), 9.7 × 10^7 PFU/ml; (B), 10.7 × 10^7 PFU/ml. A portion of sample (B) was diluted tenfold in PBS to reduce any protective light filtering effect of the NR, and
samples (A), (B), and (B, 10\(^{-1}\)) were then irradiated with visible light for 1 hour at 35° and reassayed. The titres were then (A) 10.0 \(\times 10^7\) PFU/ml, (B) 9.4 \(\times 10^7\) PFU/ml, and (B, 10\(^{-1}\)) 9.3 \(\times 10^6\) PFU/ml. In another experiment, several samples of normal virus containing 5.8 \(\times 10^8\) PFU/ml were incubated at 35° for 2 hours in PBS, pH 7.1, containing up to 400 \(\mu\)g acridine orange per millilitre, and were then irradiated for 1 hour. The maximum loss of infectivity found was 16%, which is not regarded as significant.

The Effect of the Concentration of NR and Acridine Orange on the 8-Hour Yield of Virus Grown in Darkness, and on the Light Sensitivity of this Yield

The following experiments were performed in order to determine the most suitable dye concentrations for subsequent study.

Replicate 17-hour monolayer cultures were formed as described in Materials and Methods, in the absence of NR or in the presence of either 0.4, 4, or 40 \(\mu\)g NR/ml. The cultures were then infected as described, and the original medium was returned to the culture bottles. Eight hours after addition of the virus, the cells were lysed with deoxycholate, and the lysates were assayed for infectivity. Each NR lysate was then diluted in PBS to 0.004 \(\mu\)g NR/ml and irradiated for 15 minutes at 35°.

The light sensitivity of the 8-hour yield increased with the concentration of the NR in which the virus had been grown (Table 1). The low yield in darkness at 40 \(\mu\)g NR/ml may be due to a cytotoxic effect of NR or to inadequacy of the safe light. Under these conditions, the final “virus yield” contains about 2 PFU/cell of virus inoculum which did not go through eclipse, and which therefore would be light resistant. This almost surely accounts for the high survival (28%) after irradiation of this virus preparation. The virus grown in 4 \(\mu\)g/ml of acridine orange was therefore considered suitable for further study.

The Effect of Light on Cells Growing in the Presence of NR

The following experiment was performed in order to estimate the effect of light plus NR on the rate of growth of uninfected ERK cells. Replicate monolayers were formed as described, either without NR or in 4 \(\mu\)g NR/ml. Five NR and 5 normal monolayers were trypsinized and the cells were counted at the beginning of the experiments. Three groups of 10 cultures contain-
The Growth of ERK Cells after Irradiation in Presence of Neutral Red

<table>
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<th>Hours after irradiation began</th>
<th>No NR (in darkness)</th>
<th>With NR (4 μg/ml)</th>
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<tr>
<td></td>
<td>in darkness</td>
<td>1 hour's light</td>
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<tr>
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<td>48</td>
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</table>

* Monolayers were established by 17 hours' incubation at 35° in presence (4 μg/ml) or absence of NR. All irradiations commenced simultaneously; cells were trypsinized at the indicated times after the beginning of irradiation and counted.

The Effect of Preirradiation on the Virus Yield from NR-Containing Cells

The following experiment was performed in order to estimate the effect of light plus NR on the subsequent ability of the cell to support the multiplication of poliovirus. Replicate monolayer cultures were prepared in 4 μg NR/ml. One set of such cultures was irradiated for 1 hour, a second set for 3 hours, and a third set was kept in darkness. On completion of irradiation, all three sets were simultaneously infected in darkness as described in “Methods.” Eight hours after infection, the cultures were lysed with deoxycholate and assayed for infectivity. The yields were 366, 408, and 177 PFU/cell after 0, 1, or 3 hours’ preirradiation, respectively. This indicates that NR plus 1 hour’s irradiation before infection does not damage the cells sufficiently to affect the ultimate yield of virus.

The Stage of Growth at Which the Ultimate Yield of Virus Becomes Affected by Light in Presence of NR

It has been reported previously (Wilson and Cooper, 1962) that the time in the growth process of poliovirus at which the presence of NR leads to light sensitivity is at, or at about, the time of onset of maturation. However, these experiments used 15-minute light pulses which, from 1-3 hours after infection, had been given at hourly intervals. Using this procedure, a transient period of light sensitivity occurring during the 1-3 hour period of virus growth might not have been observed. In addition, a phase of sensitivity occurring up to 1 hour after infection might have been masked by the light sensitivity of the inoculum, since NR virus had been used to infect the cultures. Figure 1 shows the results of an experiment in which normal virus was used as inoculum, together with light pulses of 1 hour’s duration given at hourly intervals. With this procedure, no portion of the growth cycle up to 8 hours escaped irradiation in the presence of NR.

The experiment was as follows. Replicate monolayer cultures formed in NR (4 μg/ml) were infected with normal virus as described in “Materials and Methods.” Figure 1, curve A, shows the course of virus synthesis in darkness, determined by harvesting one culture at hourly intervals after infection. Under these conditions, virus maturation generally began between 3 and 4 hours after the beginning of adsorption. Figure 1, curve B, shows the postirradiation course of virus synthesis in darkness in 7 cultures which had been irradiated between 5 and 6 hours, returned to darkness,
The effect of light on the growth of poliovirus in the presence of NR. Replicate monolayer cultures formed in NR solution (4 μg/ml) were completely infected with normal virus and incubated in darkness as described in the text. Curve A: production of virus in darkness, harvested at the times indicated after the addition of virus. Curve B: subsequent growth of virus in darkness, in cultures irradiated between the 5th and 6th hours after addition of virus. Curve C: 8-hour yield of cultures irradiated at various times during growth. The cultures of curve C were irradiated for 1 hour, ending at the times indicated; after irradiation the cultures were incubated in darkness and lysed at 8 hours for assay of infectivity.

and harvested at hourly intervals subsequently. Nearly all the mature progeny already formed was inactivated by the pulse of light, but some maturation of virus continued to occur in the cultures until 8 hours, although many fewer PFU were produced per unit time than in the control. The virus maturation observed after the photodynamic inactivation may be due to a small increase in virus particles in all cells or to an asynchronous maturation in a few late finishing cells. It is noteworthy that, since the majority of the mature progeny had not been released from the cell by 6 hours in this system (unpublished data), light treatment efficiently inactivated intracellular as well as extracellular NR virus.

Figure 1, curve C, shows the effect on the 8-hour yields of 1-hour pulses of light given at hourly intervals so that all phases of the growth cycle received irradiation. It can be seen that irradiation during the first 3 hours of the latent period did not affect the final virus yield to a significant extent. There was, at maximum, a decrease of 25% in these cultures compared with the
8-hour yield in the control (curve A). After this time, however, irradiation depressed the yield very much more. Thus, in the presence of NR (4 μg/ml), no portion of the growth cycle was markedly sensitive to light until at or near the first appearance of mature progeny virus.

An additional finding derived from Fig. 1 was that irradiation later than 3 hours after infection resulted in a decrease in the 8-hour yield which was greater than could be accounted for by inactivation only of mature virus formed before the termination of irradiation.

The inability of the irradiated cultures to mature as much virus after irradiation (curve B) as did control cultures (kept in darkness throughout the virus growth cycle) during this same period was consistent in all of a number of similar experiments and was reflected in the depression of the 8-hour yields in curve C. Table 3, giving the results from which curves A and C were derived, illustrates the point. Column 2 shows the presence of virus in the control cultures at various times during the virus growth cycle, column 3 the yield yet to be matured after these times. If a pulse of light did no more than inactivate virus already synthesized, then column 5, showing the amount of virus matured after irradiation, should be identical to column 3; there was, in fact, always less by the amount indicated in column 6, which was of the same order of magnitude from 1 to 6 hours after adsorption.

This might be accounted for either by: (1) the photoinactivation of a possible virus precursor; (2) the possible hastening of virus maturation during irradiation with simultaneous inactivation; or (3) damage to the cells' ability to synthesize virus. The latter possibility gains some support from the slight inhibitory effect of light applied during the first 3 hours of virus infection. Consequently, the experiments involved in curves A and C were repeated, using a short light pulse of 5 minutes which, when given up to 3 hours after infection, did not decrease the 8-hour yield. Nevertheless, 5 minutes' irradiation given later than 3 hours after infection still depressed the 8-hour yield to the same extent as did 1 hour's irradiation. The extent of this depression was again greater than would have been the case had only mature virus, already formed, been inactivated. The experiment using the 5-minute pulse thus makes the existence of a light-sensitive premature form of the virus seem more likely, but does not eliminate the alternative (1) mentioned above.

The Effect of Delaying the Addition of NR on the Proportion of Light-Sensitive Progeny in the 8-Hour Yield

The results described in Fig. 1 show that the virus-cell complex did not become light sensitive much before the time at which mature progeny appeared, as reflected by depression of the 8-hour virus yield. However, the critical reaction between NR and the virus-cell complex which ultimately led to light sensitivity might have occurred considerably earlier in the growth cycle. The time at which this reaction took place was revealed by the following experiment.

Replicate monolayers were formed in the absence of NR and infected at 35° as described. The original medium was replaced; NR was added to 4 μg/ml to one culture at the time of adding virus and to the remainder at approximately hourly intervals up to
Fig. 2. The effect on the light sensitivity of poliovirus progeny of delaying the addition of NR. Replicate monolayer cultures, formed in the absence of NR, were completely infected with normal virus and incubated in darkness as described in the text. At the intervals indicated by the abscissae, one culture received NR (to 4 μg/ml). Eight hours after infection, all cultures were lysed and assayed for infectivity (curve A). A portion of each lysate was irradiated for 15 minutes, and reassayed to determine the surviving infectivity, which is expressed as a percentage of the unirradiated yield (curve B).

7 hours later. Apart from manipulations under the safe light, incubation took place in darkness. Eight hours after adding virus, the cells were lysed by adding deoxycholate to all cultures. A portion of each lysate was irradiated for a time (15 minutes at 35°) sufficient to inactivate 97% of virus grown in continuous presence of 4 μg NR/ml, and both irradiated and nonirradiated portions of the lysates were assayed for infectivity.

Figure 2, curve A, shows that the 8-hour yield (unirradiated) was unaffected by the time of addition of NR. In contrast, curve B shows that NR rendered almost all the progeny light sensitive if added as late as 3 hours after infection. The proportion which survived irradiation increased markedly after this time. Thus the change in the virus growth process which rendered the progeny sensitive to light was associated exclusively with a late stage of growth. Furthermore, when NR was added at 7 hours, the progeny virus, which was largely intracellular, did not become light sensitive although the NR entered the cell, so that simple intracellular mixing of NR and mature virus was no more effective than extracellular mixing in conferring light sensitivity. Hackett (1962), using vesicular exanthema virus (also a RNA virus) and acridine orange found similar effects. She showed that to acquire light sensitivity, virus must be grown with the
Fig. 3. The gain of light and antiserum resistance of NR poliovirus after adsorption to cells containing no NR. A concentrated suspension of cells was infected in darkness with NR virus and washed at 0°, as described in the text; the cells were then diluted in growth medium at 35° and stirred. After the indicated intervals, 2 samples were taken: one was irradiated for 10 minutes (●), the other was treated with antiserum (○). After treatment, the surviving infective centers were assayed. The results are expressed as a percentage of the total infective center (IC) content as assayed in untreated samples.

dye and that such light sensitivity was lost soon after virus adsorption to fresh cultures.

A Comparison of the Rate of Loss of Light Sensitivity and the Rate of Gain of Antiserum Resistance of the Infective Center after Adsorption of NR Virus

Wilson and Cooper (1962) showed that NR virus rapidly lost its light sensitivity after adsorption to cells. The following experiment was done to compare the time course of this loss with that of another early stage in the growth process, namely the rate at which the infective center becomes resistant to antiserum.

A suspension of cells (5 × 10⁶/ml, pH 7) was stirred for 17 hours at 35° in medium CSV.6 without NR. The cells, after centrifugation, were resuspended at 0° to 5 × 10⁶ cells/ml in PBS containing 4 × 10⁶ PFU/ml of NR virus (this inoculum had been twice passaged through cultures containing NR, 4 µg/ml, to eliminate original light-resistant virus, before concentration, as described in Materials and Methods). The infected cells were then washed in 100 ml PBS, resuspended to 10⁶ cells/ml in CSV.6 at 35°, and gently stirred. The time at which this resuspension was effected was regarded as the start of infection. At intervals, samples were diluted (1) into chilled CSV.6 containing antiserum sufficient to inactivate 90% of free virus in 10 minutes, and then kept at 0° for 30 minutes, and (2) into chilled CSV.6 and then irradiated for 10 minutes. After these treatments, both sets of samples were assayed for infective centers.

Figure 3 shows the results of this experiment. The absorbed NR virus lost its light sensitivity at a rate almost identical with that with which the infective center became resistant to antiserum. This rate, which was obtained in the absence of free NR, was similar to that obtained in the continued presence of 4 µg/ml NR (Wilson and Cooper, 1962).
In the experiment described in Fig. 3, the suspended virus culture was maintained until 8 hours after infection, at which time a sample was lysed and diluted to $10^{-2}$ in PBS. The titre of the diluted lysate was $1.2 \times 10^5$ PFU/ml (equivalent to 313 PFU per infective center); after irradiation for 15 minutes at 35°, its titre was $1.1 \times 10^7$ PFU/ml. Thus a light-sensitive inoculum gave rise in the absence of NR to light-resistant progeny, as might well be expected.

A Comparison of the Rate of Loss of Light Sensitivity of Infective Centers Formed by NR Virus and Virus Grown in the Presence of Acridine Orange

In view of the chemical and biological similarity of neutral red and acridine orange, the following experiment was done to determine whether virus grown in presence of acridine orange also lost its light sensitivity after adsorption to cells, and whether the rate of loss was the same as that of NR virus.

Replicate monolayers were formed in petri dishes as described in the "Materials and Methods;" no dye was incorporated. The medium was removed, and about 100 PFU either of NR virus or of virus grown in presence of acridine orange (0.4 μg/ml) were added in 0.2 ml of PBS to each dish. The dishes were left for 10 minutes at 35°. (One culture of each series was irradiated during adsorption and for 5 minutes afterwards, but the remainder were handled under the safe light.) After adsorption, the cultures were washed with 5 ml cold PBS, placed on a chilled slab of metal, and overlaid with agar medium. After the agar had set and within 5 minutes of chilling, the cultures were warmed to 35° on a warm slab; the timing was kept the same for each culture. At intervals during subsequent incubation at 35°, one dish of each series was inverted and irradiated for 15 minutes, then reincubated in darkness for 3 days, when the plaques were counted as usual.

The results are shown in Fig. 4. NR virus and virus grown in the presence of acridine orange produced identical rates of gain of light resistance of the infective centres formed in the monolayers.

DISCUSSION

Ledinko (1958) showed that apparently complete but noninfective poliovirus particles were formed in presence of proflavine; these experiments were presumably carried out under normal conditions of lighting. Schaffer (1960) demonstrated the presence of proflavine in poliovirus particles when they had been synthesized, not merely suspended, in solutions of the dye; he later found (personal communication) that particles containing proflavine were inactivated by visible light. Acridine orange was shown by fluorescence also to be incorporated into poliovirus particles during growth in presence of the dye (Mayor and Diwan, 1961). Crowther and Melnick (1961) found that light did not inactivate mature poliovirus when it was mixed with NR or acridine orange, but did so when virus was grown in the presence of either; they also pointed out the structural similarities of neutral red, proflavine, and acridine orange. All these findings make it very likely that the light sensitivity of poliovirus grown in presence of NR results from the incorporation of NR into the virus particle itself.

We have also found that poliovirus is light sensitive if grown in the presence of NR and, in addition, we have shown that the time of application of light during the growth cycle is of importance. Light has little effect during the first 3 hours of the latent period. At, or just before, the time of appearance of mature NR virus, light has an increasingly large effect. Moreover the presence of NR is not required much before this time in order to render almost all the progeny virus light sensitive.

Light sensitivity of NR virus is quickly lost on adsorption to fresh cells, both in the absence and in the continued presence of NR. This loss is not due to some form of shielding by the cells, since NR virus was effectively inactivated intracellularly. The rate of loss of light sensitivity of virus virus is similar to the rate of loss of light sensitivity of virus grown in acridine orange, and to the rate of gain of resistance of the infective center to antiserum.

The lack of effect of light plus NR on the mature particle, and the close temporal
Fig. 4. The effect of light on the establishment of plaques by NR poliovirus (○) or by poliovirus grown in presence of acridine orange (●). About 100 PFU of either preparation of virus were adsorbed at 35° on replicate monolayer cultures in petri dishes, as described in the text. After adsorption, the cultures were overlaid with agar, and at intervals during incubation one culture of each series was irradiated for 15 minutes. The abscissa shows the times after the addition of virus at which irradiation began. After irradiation, the dishes were reincubated for 3 days; after staining, the plaques were counted. The plaques recovered are expressed as a percentage of the plaques obtained in unirradiated controls.

The association of the gain in light sensitivity with the onset of maturation, strongly suggest that the light sensitivity of the NR virus results only from the inclusion of NR in the particle as it matures, or in a precursor present just before maturation. Incorporation into a precursor is suggested by the experiment of Fig. 1, but the virus-cell-NR complex may become more sensitive to light just before the onset of maturation for some other reason. However, whichever is the case, it is reasonable to suppose that the disaggregation of the virus particle, which we presume must occur at least partially before replication of the RNA can commence, might be responsible for the loss of light sensitivity. For these reasons, it seems likely that the loss of light sensitivity is closely associated with some disaggregation of the poliovirus particle. The proximity in time of the gain of light and antiserum resistance indicates that such disaggregation is likely to occur at or near the cell surface.

Whatever explanation is given for the gain of light resistance, it is significant that light, unlike antiserum, is effective both inside and outside the cell. Therefore a simultaneous gain in resistance to both light and antiserum reflects more than the simple passing of the intact virus particle through an antibody impermeable cell surface, and implies that there must be some change of structure of the virus particle at or about this time.

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Interference between Polioviruses Induced by Strains that Cannot Multiply

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Poliovirus type 1 strains unable to make infective virus at 39.5° can nevertheless suppress the production of type 1 challenge strains that are otherwise able to grow at 39.5°. The majority of mixedly infected cells fail to yield any progeny at this temperature. A constant ratio of interfering to challenge viruses gives a constant degree of interference. With a constant interfering dose, the interference is less at high challenge doses. A 15-minute interval between first infection and challenge generally gives maximal interference, but the interference can be extensively reversed by high challenge doses even after an interval of 1 hour. The degree of interference varies with different cell strains and with different interfering and challenge strains. The interference results from a hindrance to a stage after adsorption and uncoating but before the formation of infective RNA. Interference is unaffected by the use of guanidine (200 µg/ml) in place of 39.5° as the selective condition. Whereas the interference between enteroviruses described by other workers may reflect some competition during replication, the interference described here must result from some event occurring before the synthesis of interfering virus RNA.

INTRODUCTION

This paper shows that strains of poliovirus type 1 that cannot grow at 39.5° or in presence of guanidine can nevertheless completely block the growth of other type 1 strains that can grow under these conditions. Most of this exclusion results from some mechanism other than a hindrance to adsorption or to penetration. The interference is less effective, however, when both strains can multiply.

Considerable interference also occurs between poliovirus types 1 and 2 and other enteroviruses under conditions that permit growth of both strains (Ledinko, 1963a; Cords and Holland, 1964). Such interference between actively multiplying strains differs, possibly in a fundamental way, from the interference described in this paper.

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MATERIALS AND METHODS

Virus strains. The derivation and some relevant properties of poliovirus strains VS (vaccine Sabin), VS.41 (vaccine Sabin adapted to grow at 41° by Dr. A. Lwoff), Brunhilde and Brunenders have been described elsewhere (Cooper, 1963). The two strains used most frequently in the present work were hd+ and hd-19; hd+ is a clone of strain VS.41 picked from a plaque formed at 40°, while hd-19 is one of 26 heat-defective mutants induced in strain hd+ by growth in 1 mM 5-fluorouracil (Cooper, 1964). The guanidine-resistant strain g/1400 is a derivative of Brunhilde and was obtained through the kindness of Dr. N. Ledinko.

Infectivity assays. Virus and “infective cells” (i.e., those infective centres that are not free virus particles) were assayed by the agar cell-suspension plaque method (Cooper, 1961), employing screw-capped bottles, the
bicarbonate-glucose medium, and strain U cells (Pohjanpelto, 1961). In some experiments (mentioned in the text) ERK cells were employed. All cells were grown in medium CSV.6 (Cooper et al., 1959). Virus able to grow at high temperatures or cells infected with such viruses were assayed in the presence of heat-defective virus or of cells infected with heat-defective virus by incubating the plaque assay bottles in a water bath for 3 days at 39.5°C; the ratio of plaques obtained at 39.5°C to those obtained at 37°C of hd-19 or of VS (or of cells infected with these viruses) were $10^{-6}$ and $10^{-4}$, respectively. The 37°C assays employed identical bottles in an air incubator. The guanidine-resistant strain g/1400 was assayed in the presence of the guanidine sensitive virus by including 200 µg guanidine hydrochloride per millilitre in the base layer and by incubating for 3 days at 37°C. This concentration of guanidine reduced the plaque count of strains VS and hd-19 to $10^{-4}$.

Infective RNA extraction and assays (Alexander et al., 1958; Koch et al., 1960) employed cold aqueous phenol and monolayers which were washed once with phosphate-buffered saline (PBS) and once with $M$ NaCl (pH 8.1). The cells were then incubated for 8 minutes at 37°C with 0.1 ml of RNA diluted in $M$ NaCl and finally washed twice with PBS. Interference tests using infective RNA as challenge used the same infection procedure.

Terms used to express interference. "Absolute plating efficiency" of an infected cell suspension means the proportion of cells that form a plaque under defined conditions, and is equal to the number of PFU/ml in washed cell suspensions when assayed at, say, 39.5°C, divided by the number of cells per millilitre. The extent of interference in cells infected with challenge plus interfering virus is expressed in terms of "relative plating efficiency," which is a measure of the decrease in infectivity caused by the interfering virus. The "relative plating efficiency" of a cell suspension undergoing interference is defined as the absolute plating efficiency (when assayed at 39.5°C or in presence of guanidine) of the cell suspension receiving both interfering and challenge viruses divided by the absolute plating efficiency of a replicate cell suspension infected with challenge virus alone.

Test of interference. Cells were always infected at 0°C, usually by agitating a cell suspension (2 X 10⁷ cells/ml) with virus for 30 minutes in an ice bath. The cells were washed once before the challenge infection, which was carried out in the same way. Some experiments involved incubation of cells in water baths at 37°C or 39.5°C between infections, in which case cells were rechilled for the second infection. After that the cells were washed twice and assayed for infectivity at 37°C and at 39.5°C. Free virus was always $<1\%$ of the total infectivity. Each experiment included controls comprising cells infected with the interfering or the challenge viruses alone; cells infected with the interfering virus alone produced negligible numbers of plaques at 39.5°C or in presence of guanidine (absolute efficiency of plating was $<10^{-4}$). When fully infected about 50% of the cells produced plaques. The multiplicities of infection were calculated from the dose of input virus.

Some experiments measured the one-step growth of virus in replicate monolayers (3 X 10⁶ cells) grown on the base of flat-bottomed tubes. In these experiments low multiplicities of challenge virus were adsorbed first at 0°C, the cells were then washed, and the interfering strain was added, still at 0°C. After a further wash, 1 ml of Eagle's medium containing 5% calf serum (with or without guanidine) was added to each tube, the tubes were immersed in a water bath at the appropriate temperature, and replicate tubes were frozen at intervals for assay of infectivity.

RESULTS

Effect of Multiplicity of Infection and Time of Challenge

Interference was tested using various multiplicities of a challenge virus (hd⁺) that could grow at 39.5°C, and a fixed multiplicity (8 PFU/cell) of an interfering virus (hd-19) that could not grow at 39.5°C. The criterion used was the ability of hd-19 to prevent plaque formation by infective
The results, given in Fig. 1, show that the degree of interference is dependent on multiplicity of challenge virus. Three findings emerge from this figure. First, the growth of small doses of \( h d^+ \) is suppressed in 70\% of the cells even if they were first infected with \( h d^+ \) and incubated for 15 minutes at 39.5\(^\circ\) before infection with \( h d-19 \). Thus extensive interference can occur even though adsorption of challenge virus is not prevented.

Similar effects were also found in ERK cells with the cell suspensions at 0\(^\circ\); a time of challenge of 0 minutes indicates that challenge and interfering virus were adsorbed simultaneously. The relative plating efficiency of the cells at 39.5\(^\circ\) equals the ratio of the plating efficiency at 39.5\(^\circ\) of cells infected with interfering plus challenge virus to that of cells infected concurrently with the same dose of challenge virus alone.

The results, given in Fig. 1, show that the degree of interference is dependent on multiplicity of challenge virus. Three findings emerge from this figure. First, the growth of small doses of \( h d^+ \) is suppressed in 70\% of the cells even if they were first infected with \( h d^+ \) and incubated for 15 minutes at 39.5\(^\circ\) before infection with \( h d-19 \). Thus extensive interference can occur even though adsorption of challenge virus is not prevented.

Similar effects were also found in ERK cells with \( h d^+ \) as interfering virus and VS.41 as challenge. In some independent experiments, the effect of order of adsorption of challenge \( h d^+ \) (0.1–0.3 PFU/cell) and interfering \( h d-19 \) (9–13 PFU/cell) was examined without the additional effects caused by interposed incubation; cultures were washed at 0\(^\circ\) between infections, which were also carried out at 0\(^\circ\). When \( h d-19 \) was adsorbed first, \( h d^+ \) was suppressed in 88\% of the cells (average of 4 experiments); when \( h d^+ \) was adsorbed first, it was suppressed in 68\% of the cells. Hence presumably the prior adsorption of large doses of \( h d-19 \) can decrease slightly the adsorption or penetration of challenge virus, but the major part of the interference is independent of these effects.

Second, the growth of high multiplicities of \( h d^+ \) was suppressed in only 50\% of the cells even if they were first infected with \( h d-19 \) and incubated for 60 minutes at 39.5\(^\circ\) before challenge. It seems that in most cases an interval of more than 15 minutes between first infection and challenge does not further increase the interference.

Third, the extent of interference can still be decreased by increasing the multiplicity of challenge virus even if the interfering virus has been allowed to develop for 60 minutes. This cannot be due to incomplete infection of the cells by \( h d-19 \) resulting from uneven susceptibility of cells to infection, since (a) \( h d-19 \) kills 100\% of the cells at 39.5\(^\circ\) (unpublished data), (b) identical conditions of infection permitted 99\% interference (equivalent to at least 99\% infection by \( h d-19 \)) if the challenge \( h d^+ \) multiplicity was 0.1 (Fig. 1), and (c) increasing the multiplicity of \( h d^+ \) above 4 (see Fig. 1) did not increase the relative plating efficiency above unity, an indication that all potentially susceptible cells had been infected by this multiplicity of challenge virus. Neither is the increased number of plaques at large doses of \( h d^+ \) reported in Fig. 1 caused by

**Table 1**

<table>
<thead>
<tr>
<th>Multiplicity of ( h d-19 )</th>
<th>Multiplicity of ( h d^+ )</th>
<th>Relative plating efficiency of cells at 39.5(^\circ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>4</td>
<td>0.19</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>0.14</td>
</tr>
<tr>
<td>32</td>
<td>16</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* The cells were infected with \( h d-19 \), washed, and incubated for 15 minutes at 39.5\(^\circ\) before superinfecting with \( h d^+ \).
TABLE 2

<table>
<thead>
<tr>
<th>Interfering virus</th>
<th>Challenge virus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>VS.41</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hd&lt;sup&gt;+&lt;/sup&gt;</td>
<td>g/1400</td>
</tr>
<tr>
<td>Simultaneous</td>
<td>After 30 min</td>
<td></td>
</tr>
<tr>
<td>hd-19 in U cells</td>
<td>0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.06</td>
</tr>
<tr>
<td>VS in U cells</td>
<td>0.78</td>
<td>0.85</td>
</tr>
<tr>
<td>VS in ERK cells</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Simultaneous</td>
<td>After 30 min</td>
<td></td>
</tr>
<tr>
<td>hd-19 in U cells</td>
<td>0.005</td>
<td>0.42</td>
</tr>
<tr>
<td>VS in U cells</td>
<td>0.31</td>
<td>1.0</td>
</tr>
<tr>
<td>VS in ERK cells</td>
<td>—</td>
<td>0.26</td>
</tr>
<tr>
<td>Simultaneous</td>
<td>After 30 min</td>
<td></td>
</tr>
<tr>
<td>hd-19 in U cells</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td>VS in U cells</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Simultaneous</td>
<td>After 30 min</td>
<td></td>
</tr>
<tr>
<td>hd-19 in U cells</td>
<td>0.008</td>
<td>—</td>
</tr>
<tr>
<td>VS in U cells</td>
<td>0.40</td>
<td>—</td>
</tr>
<tr>
<td>VS in ERK cells</td>
<td>0.26</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> The multiplicities of the interfering viruses were about 10 and those of the challenge viruses about 0.1 PFU/cell.
<sup>b</sup> Adsorbed either simultaneously with, or after 30 minutes' incubation at 39.5° with, interfering virus.
<sup>c</sup> The figures in the table equal the relative plating efficiencies at 39.5°.

The unclipsed hd<sup>+</sup> particles which elute to form a plaque. This can be deduced from the results recorded in Table 1, which show that the degree of interference depends upon the ratio of challenge to interfering multiplicities rather than on the multiplicity of infection of challenge alone.

**Interference between Different Poliovirus Type 1 Strains**

Table 2 shows the interference between two heat-defective and three nondefective poliovirus type 1 strains. The growth of all nondefective strains was suppressed to some extent by both defective strains, particularly if the cells were incubated with the interfering virus for 30 minutes before challenge. However, the degree of interference can vary markedly with the strain used as interfering virus. The interference also varied with the cells used for virus growth; strain VS was much more effective in ERK cells than it was in U cells.

**Lack of Effect of Interfering Virus on the Irreversible Eclipse of Challenge Virus**

In order to see whether the interference with challenge virus occurred during a step in its penetration, the eclipse of VS.41 was examined when adsorbed with VS to ERK cells. In this system, strain VS is much more effective as an interfering agent than it is in U cells (Table 2). Treatment with pH 2.5 buffer was used to recover the infectivity (Holland, 1962; Fenwick and Cooper, 1962). Figure 2 shows that the interfering virus does not prevent the challenge virus from entering acid-irreversible eclipse at a normal rate. Identical results were obtained in U cells with hd<sup>+</sup> as challenge and hd-19 as interfering virus using 8M urea (Holland, 1962) to recover the infectivity.

**Use of Infective RNA as Inoculum**

The concept that the development of the
from the interfered culture was 0.12, a value similar to the interference of hd+ intact virus by hd-19 with comparable conditions of infection. This experiment serves to illustrate two points: firstly, that interference at 39.5° by hd-19 prevents the synthesis of hd+ RNA, and secondly, that hd-19 makes much less infective RNA at 39.5° than does hd+.

**Effect of Guanidine on the Interference**

Guanidine effectively blocks the growth of poliovirus (Rightsel et al., 1961; Crowther and Melnick, 1961), and guanidine-resistant strains are available (Ledinko, 1963b). To see whether the event leading to interference was sensitive to guanidine, experiments were carried out using Ledinko's g/1400 guanidine-resistant strain as challenge and hd-19 as interfering virus. Preliminary experiments showed that incubation either at 39.5° or in 200 μg guanidine per millilitre reduced the plating efficiency of strain hd-19 to less than 10-4 of the value at 37°, whereas the same incubation conditions had no effect on strain g/1400. Thus it is possible to measure the interference with g/1400 by hd-19 using either high temperatures or guanidine as the condition selective for g/1400. Table 4 shows that hd-19 interfered with g/1400 to an equal extent under both selective conditions.

**TABLE 4**

**INTERFERENCE WITH POLIOVIRUS STRAIN g/1400 BY STRAIN hd-19 USING INCUBATION EITHER IN GUANIDINE OR AT 39.5° AS A SELECTIVE TREATMENT**

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Relative plating efficiency of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 39.5°</td>
</tr>
<tr>
<td>1</td>
<td>0.012</td>
</tr>
<tr>
<td>2</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* Cells were infected at 0° with hd-19 (multiplicity of 10 PFU/cell), incubated for 30 minutes either at 39.5° or at 37° in presence of 200 μg guanidine hydrochloride per millilitre and superinfected at 0° with g/1400 (multiplicity of 0.1 PFU/cell) in presence of guanidine where appropriate. After washing, the cells were assayed for infectivity at 39.5° or at 37° in presence of 200 μg guanidine hydrochloride per millilitre.
Comparison of Interference with and without the Multiplication of Interfering Virus

Most poliovirus interference previously reported (Ledinko and Hirst, 1961; Ledinko, 1963a; Cords and Holland, 1964) was between different serological types, whereas the work described above used only type 1 poliovirus. As discussed below, the properties of the two forms of interference differ: in particular, the homologous form appears more effective. The two sets of results cannot be compared directly, however, because the heterologous interfering strains could multiply under the conditions used, whereas our strains could not. The following experiments were therefore done to see whether the dissimilarities result from the use of heterologous strains, or from the prevention of multiplication of the interfering virus.

One-step growth of low multiplicities of g/1400 was measured in replicate monolayer cultures in presence and absence of guanidine hydrochloride (400 μg/ml), and with and without superinfection by high multiplicities of poliovirus type 2 (strain MEF). Thus heterologous interference was directly com-

Figure 3 shows that no detectable interference with g/1400 occurred under the conditions used when the MEF was able to replicate. This confirms the results of Cords and Holland (1964). However, prevention of replication of MEF with otherwise identical conditions produced significant interference. For purposes of comparison, similar experiments were done with hd-19 as interfering virus (20 PFU/cell) and hd+ as challenge (3 PFU/cell); incubation at 39.5° was used in place of guanidine to prevent replication of hd-19, and the growth of hd+ was measured by assay at 39.5°. Figure 4 shows that while some interference occurred at 37° with the doses used, interference was again more marked when the interfering strain could not multiply. Hence the differences between the two forms of interference are more likely to result from the presence or absence of multiplication of the interfering virus than from the use of heterologous strains.

A block in growth per se is not sufficient to induce interference: some strains are less
effective interfering agents (see above), and many heat-defective mutants of poliovirus can neither multiply nor induce any interference at 39.5° (Cooper, unpublished results). Thus the heat defect in \textit{hd}-19 (and presumably at least one of the defects induced by guanidine) is specifically related in some way to the interference phenomenon.

Other experiments like that of Fig. 4 showed that the degree of interference at 37° by \textit{hd}-19 with \textit{hd}°, and by the heat-defective Brunhilde with the nondefective Brunhilde, increased with the ratio interfering dose:challenge dose. When \textit{hd}° was the interfering strain (10 PFU/cell) and \textit{hd}-19 the challenge (0.1 PFU/cell), the 8 hour (37°) yield of \textit{hd}-19 from the mixed infection was about 10% of its yield when grown alone. These experiments, in which both strains can multiply, all support the concept that such interference reflects some competition during multiplication.

\section*{Discussion}

Given the right multiplicity of infection, certain poliovirus type 1 strains can completely suppress the production of a homologous virus in nearly all cells, even though the suppressor strains cannot complete their own growth process. Large interfering doses may decrease the adsorption of challenge virus somewhat, but there is clearly another and later mechanism which accounts for most of the interference described in this paper. That the challenge virus is uncoated is shown by its normal eclipse kinetics and by failure to bypass the interference on challenge with infectious RNA. However, the growth of the challenge virus is arrested at some stage before its RNA synthesis.

As already mentioned, the differences between interference caused by heterologous multiplying strains and that caused by homologous nonmultiplying strains result more from the presence or absence of multiplication than from the use of dissimilar strains. It is convenient now to list these differences.

First, when the interfering virus can multiply, more than 30 minutes must elapse between interfering and challenge doses in order to produce any interference. When multiplication is prevented, certain challenge doses added even before the interfering virus are strongly interfered with. Second, when the interfering virus can multiply and 60–80 minutes elapse between interfering and challenge doses, then the interference cannot be reversed even by high challenge doses. When multiplication is prevented, the interference remains reversible by high challenge doses up to this time. Third, and perhaps most important, prevention of multiplication of heterologous interfering virus with guanidine 1 to 2 hours \textit{after} infection completely reverses the interference (Cords and Holland, 1964). In our homologous and heterologous cases, treatment with guanidine or high temperature from the beginning of infection allows interference.

These properties of interference by strains that can multiply strongly support the suggestion (Cords and Holland, 1964) that this interference reflects some event of a later stage of growth, perhaps a competition for replicating sites or for the finite resources of the cell. Our experience with multiplying strains is similar. In contrast, the interference induced in the absence of multiplication is likely to reflect some earlier event.

In any case, the two forms appear to differ qualitatively and are likely to be initiated by different events. Presumably the event causing interference in the absence of multiplication occurs even before the interfering virus has induced RNA polymerase and made its RNA, since guanidine prevents these syntheses (Baltimore \textit{et al.}, 1963) but allows interference. Additional evidence for this is that \textit{hd}-19 cannot make infective RNA (or coat antigen, unpublished data) at 39.5°.

Experiments to test the effect of puromycin on the interference were inconclusive, presumably because of the reversible nature of its action. The apparently rapid onset of interference coupled with its failure to increase with longer incubation makes a contribution by interferon seem unlikely, but too little is known of interferon action to say much at present.

Two simple models (which are not mutually exclusive) might account for the properties of the interference induced in the
absence of multiplication, in particular the property of reversibility of interference by high doses of challenge virus. The first proposes that the two RNA's vie for a site used before viral RNA synthesis. The interfering RNA either may irreversibly occupy the more accessible of such sites, leaving the less accessible for challenge RNA, or may reversibly compete with challenge RNA, in which case the interference may follow simple mass action laws.

The second model proposes that specific proteins (coded for either by virus or by host) are induced by the interfering virus, and these prevent the replication of challenge RNA. Conceivably, either (a) the entry of viral nucleic acid always induces interferon production (Rotem et al., 1963) and viral genes consequently needed to counter its action are defective in k-19 or in presence of guanidine, or (b) viral gene products exist which destroy heterologous polyribosomes (Penman et al., 1963), or (c) polymeric viral proteins may be rendered defective by the phenotypic mixing of competent and defective monomers produced by the two competing viral genomes.

ACKNOWLEDGMENTS

We are grateful to Dr. Nada Ledinko for the guanidine-resistant strain, to Mr. A. Bellett for help in preparation of infectious RNA, and to Miss Ann Marden for technical help.

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REFERENCES


The Effect of Light on Poliovirus Grown in Neutral Red

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The rate of inactivation by white light of poliovirus grown in neutral red (NR) solutions is decreased by a lowering of NR concentration and of oxygen tension and, to a small extent, of temperature. Photoinactivation entails no gross change in the structure of the virus or the contained RNA, but results in loss of infectivity of the RNA moiety of the virus. White light does not reduce the infectivity of mixtures of NR and free (infectious) RNA, and free infectious RNA derived from NR virus is also light resistant.

INTRODUCTION

Poliovirus grown in solutions of certain acridine dyes or of neutral red (NR) is inactivated by white light (Schaffer, 1962; Crowther and Melnick, 1961; Wilson and Cooper, 1962). No inactivation occurs if normal poliovirus is merely mixed with the dyes, and the acridine compounds, which are chemically very similar to NR, are incorporated into the particle itself during virus synthesis (Schaffer, 1962; Mayor and Diwan, 1961). It is presumed in the work described here that NR is also incorporated in the virus particle.

When poliovirus is grown in NR solutions, sensitivity to light is gained at or just before virus maturation (Wilson and Cooper, 1962). However, such light-sensitive virus becomes light resistant within an hour of infecting fresh cells, even if the NR is still present in the medium, and the kinetics of gain of resistance to light and to antiserum are closely similar (Wilson and Cooper, 1963).

This paper examines the nature of some changes in the light-sensitive poliovirus particle caused by photoinactivation, and suggests a model to explain the fact that NR confers light sensitivity upon poliovirus only when it is confined within the particle.

MATERIALS AND METHODS

It is convenient to refer to virus grown in NR solutions as "NR virus." Unless otherwise stated this means virus grown as described below in 4 µg NR per milliliter. Except during the irradiations described in the text, all incubations involving NR or NR virus took place strictly in darkness; manipulations were performed only under a red safe-light, and as rapidly as possible.

Irradiations took place at 36°, 46 cm below an 80-watt "daylight" fluorescent strip (Wilson and Cooper, 1963).

Cells. ERK cells grown in medium CSV.6 (Cooper et al., 1959) were used throughout.

Virus and infective cell assays employed the agar cell-suspension plaque assay (Cooper, 1961). "Infective cells" means those cells able to yield a plaque when plated in the same way as free virus. Their infectivity was shown not to be due to contaminating free virus.

Infective RNA was extracted by phenol and assayed as described by Cooper (1962). Neutral red was supplied by Hartman-Leddon Co., Philadelphia, Pennsylvania;
the colour index was number 825, and the total dye content was given as 92%. Phenol red was omitted from all media.

**Growth and purification of virus stocks.** All virus used was poliovirus type 1 (Brunendorf strain). (1) Normal virus: Cells were resuspended to $10^8$/ml in 5 ml PBS (pH 7.0) containing $5 \times 10^7$ PFU/ml and left for 15 minutes at 35°, then diluted into 95 ml growth medium CSV6 and stirred at 35° for 8 hours; the culture was then lysed with 2 mg sodium deoxycholate per milliliter. The lysate was clarified by centrifugation at 10,000 rpm for 15 minutes in the Spinco 30 rotor, and the supernatant was centrifuged at 40,000 rpm for 90 minutes in the Spinco 40 rotor to sediment the virus. The sediment was allowed to resuspend overnight at 4° in 0.02 M phosphate (pH 6.8), made up to 2 ml and washed through a 10-ml DEAE column (Hoyer et al., 1959) prepared in 0.02 M phosphate. The eluate was made up to 11 ml in 0.02 M phosphate and given two cycles of washing by centrifugation at 40,000 rpm for 90 minutes followed by a clarification at 10,000 rpm for 15 minutes, both in the Spinco 40 rotor. (2) NR virus: The above procedure was followed except that 4 µg NR per milliliter was present throughout the growth cycle and the inoculum was itself NR virus. (3) P32-labelled NR virus: The procedure was as for NR virus except that adsorption and growth took place in the nutrient solution of Marcus et al. (1956), from which phosphate had been omitted and replaced by P32-carrier free phosphate to 20 µc/ml. The purity of the labelled virus was established by centrifugation through CsCl and sucrose gradients.

**Sucrose density gradients.** NR virus and infective RNA preparations were centrifuged through sucrose gradients as follows. The gradients covered the range 90 to 300 mg sucrose per milliliter in 0.01 M Versene; the samples in 0.2 ml were carefully layered on top of the gradients, which were then centrifuged at 35,000 rpm, for 1 hour or 4.5 hours for NR virus and infective RNA, respectively, in the SW/35 swingout head of the Spinco model L centrifuge. The resultant partly sedimented preparations were taken from each tube by collecting fractions of 2 or 3 drops from a pinhole made in the bottom of the tube.

**RESULTS**

### Conditions Affecting the Rate of Photoinactivation of NR virus

The effect of NR concentration. Replicate monolayers of ERK cells formed in 0, 0.13, 0.4, 1.3, or 4 µg of NR per milliliter were completely infected with poliovirus (as shown by infective centre assay), and the noneclipsed virus was neutralized with a pulse of antiserum between 1.5 and 2.5 hours after infection. After washing, incubation was continued in the same concentration of NR. Virus was harvested at 8 hours by disrupting the cells with 2 mg sodium deoxycholate per milliliter (yields were 175 to 210 PFU/cell in all cultures), diluted tenfold in PBS at 36°, and irradiated. Samples were diluted at intervals in cold PBS for assay; the percentages of survivors are shown in Fig. 1.

Figure 1 shows that the light sensitivity of the virus is markedly dependent on the NR concentration in which it is grown. The absence of a shoulder near the origin suggests that the absorption of a single photon is sufficient to destroy the infectivity. In addition, there appears to be a light-resistant fraction, the proportion of which is inversely...
PHOTOINACTIVATION OF POLIOVIRUS

TABLE 1
THE EFFECT OF OXYGEN ON PHOTOINACTIVATION OF NR POLIOVIRUS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Under nitrogen</th>
<th>Under oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PFU/ml X10^2</td>
<td>Per cent survival</td>
</tr>
<tr>
<td>At beginning of irradiation</td>
<td>345 (100)</td>
<td>325 (100)</td>
</tr>
<tr>
<td>After 10 minutes in light</td>
<td>96  27.8</td>
<td>1.05  0.32</td>
</tr>
<tr>
<td>After 10 minutes in darkness</td>
<td>320  92.8</td>
<td>345  106.1</td>
</tr>
</tbody>
</table>

*Virus grown in 4 µg NR per milliliter was diluted 10^{-4}-fold either into 0.02 M phosphate (pH 6.8) which had been refluxed for 1 hour under N_2 and cooled, or into the same solution which had been regassed with O_2. The dilutions were irradiated at 36° in sealed bottles which had been gassed with N_2 or O_2, respectively.

related to the NR concentration. Similar results have been found by Schaffer (1962) with poliovirus grown in proflavine solutions.

The effect of temperature. The virus grown in 4 µg NR per milliliter, whose inactivation characteristics are shown in Fig. 1, was diluted fiftyfold in PBS, assayed, and dispensed into screw-capped bottles. One bottle was submerged in water at 34°, the other in water at 2°; both bottles were irradiated for 5 minutes at the same distance from the light source. The survival of infectivity at 34° was 3.3%, and at 2° was 12.5%. A similarly small effect of temperature was found by Schaffer (1962) with poliovirus grown in proflavine.

The effect of oxygen. Table 1 shows that the photoinactivation of NR virus is greatly enhanced by the presence of oxygen. Oxygen has no effect on the survival of NR virus in darkness. Schaffer (1962) found that oxygen similarly influenced the photoinactivation of poliovirus grown in proflavine.

The Effect of Photoinactivation on the Properties of NR Poliovirus

The sedimentation pattern of photoinactivated NR virus. A sample of purified NR virus labelled with P^32 (see Materials and Methods), containing 5 × 10^8 PFU/ml and 7500 counts per minute per milliliter (cpm/ml) in 0.02 M phosphate, was mixed with ribonuclease to give 30 µg of enzyme per milliliter. (This was done in order to examine irradiated virus under conditions resembling those usually obtaining during assay of the virus, since it was possible that the change in structure occurring during irradiation led to inactivation of the virus only when RNase was present. If this were the case, it is supposed that the primary effect of irradiation would be to damage the capsid so as to admit the enzyme.) One half of the virus plus RNAse was then irradiated (to <10^{-4} survivors) for 10 minutes at 36°, while the other half was kept for 10 minutes at 36° in darkness. Samples of 0.2 ml of each portion were centrifuged through sucrose density gradients as described in the Materials and Methods section, and drops taken from the bottom of the tube were assayed for infectivity (control only) and radioactivity. Figure 2 shows the results of this experiment: the peak of P^32 from the irradiated sample coincides with the peaks of infectivity and P^32 from the nonirradiated control. Hence, despite the presence of ribonuclease, irradiation causes neither a detectable change in the density of the particle, nor an appreciable loss of P^32.

Adsorption of photoinactivated NR virus to
The adsorption rate of irradiated NR virus was determined as follows. Purified \(^{32}P\)-labelled NR virus was diluted into warm PBS. One half of this dilution was irradiated for 20 minutes at 36° (leaving \(10^{-3}\) survivors), while the other half was kept in darkness under the same conditions. Both portions were then chilled to 0° and assayed for infectivity; the remainder of the experiment was done at 0° using chilled materials. Washed ERK cells in 1 ml of cold PBS were mixed with 1 ml of each portion of virus to give 906 cpm/ml and \(5 \times 10^7\) cells/ml, and the adsorption mixtures were rocked gently. The infectivity of the cell suspension containing irradiated virus was 2.8 \(\times 10^9\) PFU/ml, while that of the nonirradiated suspension was 3.3 \(\times 10^8\) PFU/ml. Cell samples taken at various times after mixing were washed 3 times with 10 ml PBS, and finally resuspended in 2 ml PBS. This suspension was assayed for infective cells and for \(^{32}P\), and the results are expressed as \(^{32}P\) adsorbed and infective cells formed per milliliter of adsorption mixture (Fig. 3).

Figure 3 shows that there was no significant difference between the rates of adsorption of \(^{32}P\) from irradiated and nonirradiated NR virus suspensions. As is to be expected, the rates of \(^{32}P\) adsorption were indistinguishable from the rate of infective cell formation in the irradiated sample, in which all infective cells resulted from adsorption of a single surviving virus particle. In the nonirradiated control, in which multiple infection occurred, the formation of infective cells (not shown in Fig. 3) was essentially complete by 10 minutes, when the ratio of infective cells to total cells was about 0.2.

The following experiment shows that the irradiation of labelled NR virus after its adsorption to cells caused no release of \(^{32}P\) into the medium. The entire experiment was carried out at 0° to prevent both normal sloughing and normal eclipse of virus (Fenwick and Cooper, 1962). The labelled NR virus was rocked gently with cells at 0° for 1 hour, in a suspension containing \(5 \times 10^7\) cells, \(1.7 \times 10^8\) PFU, and 1100 cpm/ml; the cells were then removed, washed, and resuspended in CSV.6. One half of this suspension was irradiated for 10 minutes, while the other half was kept in darkness. Each half was assayed for infective cells, then the cells were removed by centrifugation and the various fractions were assayed for \(^{32}P\) and infectivity. The results are given in Table 2. Whereas irradiation reduced the infective

![Figure 3](image_url)

**Figure 3.** Comparison of the rate of adsorption of \(^{32}P\) and the rate of infective cell formation, using irradiated and nonirradiated NR poliovirus labelled with \(^{32}P\). \(\bigcirc = \text{P}^{32}\) adsorbed from irradiated virus, \(\bigtriangleup = \text{P}^{32}\) adsorbed from the same NR virus preparation kept in darkness, \(\bullet = \text{rate of infective cell formation in the irradiated preparation, representing the rate of adsorption of single virus particles.}

**TABLE 2**

<table>
<thead>
<tr>
<th>Component of suspension</th>
<th>Content per milliliter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kept in darkness</td>
</tr>
<tr>
<td>Cells</td>
<td>(10^6)</td>
</tr>
<tr>
<td>Infective cells</td>
<td>(2.2 \times 10^5)</td>
</tr>
<tr>
<td>Infectivity of supernatant</td>
<td>(3.0 \times 10^4)</td>
</tr>
<tr>
<td>(^{32}P) in supernatant (cpm)</td>
<td>0.1</td>
</tr>
<tr>
<td>(^{32}P) in cell fraction (cpm)</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*NR poliovirus, labeled with \(^{32}P\), was adsorbed in darkness at 0° to ERK cells, which were then washed thoroughly. A portion of this suspension was irradiated, and the content of infective cells and distribution of \(^{32}P\) were determined.*
The infectivity of RNA derived from irradiated NR poliovirus. The NR virus was diluted to $10^8$ PFU/ml in 0.02 $M$ phosphate (pH 6.8) and irradiated at 36°. Samples were taken before and at intervals during irradiation for assay of viral infectivity (●), and for phenolic extraction of infective RNA (○). Both types of infectivity assay were done under a red safe-light, and results are expressed as a percentage of the titres obtained from the unirradiated samples.

Inactivation of the RNA moiety. In order to see whether irradiation of NR virus damaged the RNA, samples were taken from a preparation of purified NR virus during irradiation: one portion of each sample was assayed for intact virus infectivity, and the rest was extracted with phenol. The phenol extracts were assayed for infective RNA as described in Materials and Methods. Figure 4 shows that the infectivity of the RNA derived from the irradiated virus declines pari passu with that of the virus itself. Hence infective RNA cannot be extracted from photoinactivated NR virus.

The following experiment shows that the low infectivity in the phenol extracts after irradiation is not due to failure to extract the RNA. Purified NR virus, labelled with $P^{32}$ and in 0.02 $M$ phosphate plus 0.01 $M$ Versene at 36°, was divided into two parts. One part was irradiated for 10 minutes at 36° while the other was kept in darkness. After assaying for infectivity, both portions were extracted with phenol and the extracts were assayed for infective RNA and for $P^{32}$. Table 3 shows that the specific infectivity of the extract from irradiated virus was much lower than that of the nonirradiated material. The somewhat low recovery of $P^{32}$ in the irradiated compared with the unirradiated control is attributed to manipulative losses.

It is concluded from these two experiments that photoinactivation of NR virus in all probability results from some damage to the RNA moiety of the virus.

### Table 3

The Lack of Effect of Irradiation of NR Poliovirus on the Ability of Phenol to Extract the RNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Suspension before extraction</th>
<th>Suspension after extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PFU/ml $P^{32}$ (cpm/ml)</td>
<td>Specific activity (PFU/cpm)</td>
</tr>
<tr>
<td>Before irradiation</td>
<td>$3.6 \times 10^8$ 3175 1.13 $\times 10^4$</td>
<td>— — —</td>
</tr>
<tr>
<td>After 10 minutes' irradiation at 36°</td>
<td>$5.0 \times 10^4$ 3175 1.58 $\times 10^3$</td>
<td>$1.3 \times 10^3$ 2215 0.59</td>
</tr>
<tr>
<td>After 10 minutes' in darkness at 36°</td>
<td>$3.6 \times 10^4$ 3175 1.13 $\times 10^4$</td>
<td>$6.8 \times 10^3$ 3053 223</td>
</tr>
</tbody>
</table>

* Purified NR poliovirus labelled with $P^{32}$ was extracted with phenol before and after irradiation, as described in the text.
Comparison of the sedimentation rates in sucrose gradients of RNA extracted from P32-labelled NR poliovirus. The virus, in the presence of cell lysate, either had been irradiated (O) or had been kept in darkness (●) before extraction with phenol. The third curve (-----) shows the distribution of the P32 when a portion of the RNA from the unirradiated labelled NR virus was mixed with cell lysate before centrifuging through a sucrose gradient.

The Sedimentation Pattern of RNA Extracted from Photoinactivated NR Virus

In order further to test the possibility that the inactivation of intact NR virus might really result from postirradiation damage to the RNA by RNase or other enzymes present during assay, purified P32-labelled NR virus was mixed with cell lysate (10⁶ cells and 2 mg sodium deoxycholate in 1 ml CSV.6 added to 7 ml NR virus). One sample was photoactivated at 36° whilst the other remained in darkness at the same temperature. RNA was then extracted from both samples by cold phenol treatment and assayed for infectivity; the dark sample yielded 0.16% infectivity of the original NR virus, whilst the irradiated sample yielded 0.00012%. A sample of each RNA preparation was centrifuged through a sucrose density gradient; both gave similar patterns (Fig. 5), showing that no major disruption had been caused by irradiation of whole NR virus in the presence of cell lysate. The third curve shows the marked effect of lysate when added to infective RNA after phenol treatment; when assayed after the addition of lysate, no infectivity was demonstrable.

The Effect of Light with and without NR on Free Infective RNA

The results of a previous section indicate that the part of the NR virus which is vulnerable to light is the RNA. The following experiment was done to see whether RNA extracted from nonirradiated NR virus was light sensitive. In this experiment NR virus at 3.4 × 10⁹ PFU/ml was extracted with cold phenol. One portion of the extract was irradiated at 0° for 15 minutes, whilst another remained in darkness. Assay for infective RNA showed that the irradiated sample contained 7.8 × 10⁹ PFU/ml, while the unirradiated control contained 7.7 × 10⁹ PFU/ml. Hence the extraction of infective RNA from NR virus completely removed its light sensitivity.

It was next of interest to see whether the addition of NR to infective RNA solutions restored the light sensitivity. Accordingly, normal virus at 5 × 10⁹ PFU/ml in 0.02 M phosphate was treated with cold phenol, and NR was added to some portions of the extract as indicated in Table 4. After standing for 30 minutes at 0°, one half of each portion was irradiated for 10 minutes, and all samples were then assayed for infective RNA.

Table 4 shows that, whether infective

<table>
<thead>
<tr>
<th>NR (µg/ml)</th>
<th>Irradiation (minutes)</th>
<th>Infective RNA after treatment PFU/ml</th>
<th>Percentage survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.3 × 10⁴</td>
<td>(100)</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0.70 × 10⁸</td>
<td>54.7</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>0.75 × 10⁸</td>
<td>59.2</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>4.2 × 10⁹</td>
<td>0.033</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>6.0 × 10⁹</td>
<td>0.047</td>
</tr>
</tbody>
</table>

*Portions of an infective RNA preparation were mixed with 0, 4, or 40 µg NR per milliliter and kept at 0° in darkness for 30 minutes. Samples of each NR-containing portion were then irradiated at 0° for 10 minutes while the remainder were kept in darkness, after which all samples were assayed for infective RNA.
RNA is incubated with 4 or 40 μg NR per milliliter, the action of light causes no additional loss of infectivity. The NR is demonstrated to have combined with the RNA by the inactivation in darkness of the RNA to about 50% survivors in 4 μg NR per milliliter, and to 3 × 10^{-4} survivors in 40 μg NR per milliliter. The extraction efficiency of 2.5 × 10^{-4} (1.3 × 10^{6} RNA PFU/ml from 5 × 10^{9} intact virus PFU/ml) is that which is usual in our hands.

A similar experiment, carried out at 20°, yielded similar results.

**DISCUSSION**

The experiments described above show that photoinactivation of mature NR virus is accompanied by, and very probably results from, inactivation of the RNA. This reaction is greatly enhanced by the presence of oxygen; it proceeds more slowly at lower temperatures, but the temperature coefficient is small. Neither the structure of the virus particle nor the sedimentation characteristics of the RNA are grossly changed. Infective RNA derived from NR virus is light resistant, but free infective RNA is still light resistant if NR is subsequently mixed with it. In an earlier paper (Wilson and Cooper, 1962), it was also shown that the presumably uncoated viral RNA present during the eclipse phase is insensitive to light in the presence of NR, the virus being light sensitive only before penetration and after maturation.

Those compounds of the acridine series which are chemically most similar to NR absorb visible light and readily undergo photoreduction (Millich and Oster, 1959). Their molecules are planar, and the group capable of oxidoreduction is held rigidly on the side of the molecule opposite the amino groups, which are the groups likely to be bound by the phosphate radicals in nucleic acids (Beers et al., 1958). The reactive group of the dye might thus be sterically prevented from reacting with the RNA in some circumstances.

Failure to induce light sensitivity when infective poliovirus RNA is mixed with NR seems at variance with the results both of Chessin (1960), who found that infective RNA from tobacco mosaic virus became photosensitive when mixed with 3 μg acridine per milliliter, and of Hatt (1960), using poliovirus RNA with 6 μg toluidine blue per milliliter. In the case of the former, the reaction was accomplished in 0.1 M phosphate whilst the latter used the high molar salt technique of Koch et al. (1960); our results were obtained in 0.02 M phosphate. The molarity of salt solutions apparently reversibly affects the degree of coiling of the helical structure of RNA (Doty et al., 1959; Fraenkel-Conrat, 1959), as well as its sedimentation characteristics (Warner et al., 1963). Morthland et al. (1954) and Yamamoto (1958) suggested that photosensitivity required the reaction of both amino groups of the dye with phosphate radicals of nucleic acid; it is possible, therefore, that such a condition would be fulfilled only when the poliovirus RNA is in a suitably tightly coiled form, such as may occur in the mature virus or in solutions above a certain ionic strength. Our data indicate that photoinactivation, if this indeed be the reaction undergone by poliovirus RNA, causes little fragmentation of the nucleotide chain.

Since this work was completed, Wallis and Melnick (1963) have shown that, contrary to the findings of Hatt (1960), Crowther and Melnick (1961), Schaffer (1962), and Wilson and Cooper (1962, 1963), mature poliovirus can be reversibly photosensitised by simple mixing with 10^{-4} M heterotricyclic dyes, but only when highly purified and in 0.1 M phosphate buffer, pH 8.0, although not in Tris buffer. Under these conditions, the virus-dye complex adsorbs to cationic columns, unlike the virus alone, which readily elutes. Reduction of pH causes loss of photosensitivity.

If photoinactivation entailed inactivation of the RNA alone, as suggested by our results, one would need to postulate a reversible increase in permeability of the virus particle to the dye, due perhaps to high pH. Alternatively, the phenomenon of Wallis and Melnick may involve not the viral RNA at all, but rather inactivation of the protein coat. Thus, Weil et al. (1951, 1953) showed that light in the presence of methylene blue caused the photooxidation of...
tyrosine, tryptophan, histidine, methionine, and cystine; it also caused the inactivation of chymotrypsin activity at a rate similar to the rate of photooxidation of histidine in the chymotrypsin molecule (which they suggested was one of the active centres of union between enzyme and substrate). Weil and Seibels (1955) demonstrated similar effects with ribonuclease in 0.2 M phosphate, pH 8.0, again accompanied by photooxidation of histidine. Moreover, Cummings (1963) showed that the conversion of the long-head form to the short-head form of bacteriophage T2 in Tris buffer, pH 8.5, and 10^-4 M methylene blue was prevented by irradiation, likewise, accompanied by the photooxidation of histidine.

It would be of interest to know whether isotopically labelled poliovirus, photoinactivated by the method of Wallis and Melnick, could yet adsorb to cells and whether such inactivated virus still contained infective RNA.

ACKNOWLEDGMENTS

We wish to acknowledge with gratitude the capable assistance of Miss Hilary Belham.

REFERENCES


The Study of Cell Rupture in *Staphylococcus aureus*

BY P. D. COOPER

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**SUMMARY:** In order to prepare staphylococcal cell walls as free as possible from intact organisms yet with minimum chemical and mechanical degradation, a rapid turbidimetric method was developed to measure the proportion of staphylococci ruptured by shaking with glass beads. The assay took less than a minute, and was used to find the bacterial concentration and speed and time of shaking which gave the biggest yields of cell walls in the shortest time.

When bacterial suspensions are shaken with small glass beads the turbidity decreases (Curran & Evans, 1942) and the viable count steadily drops according to a logarithmic law (King & Alexander, 1948). This appears to be due to mechanical rupture of the organisms, as the cell walls of the burst bacteria can be separated from the soluble cytoplasmic contents by centrifugation (Cooper, Rowley & Dawson, 1949; Salton & Horne, 1951). It has recently been found (Few, Cooper & Rowley, 1952) that when penicillin-sensitive staphylococci are rapidly disrupted in a medium containing formalin ('Medium A', see below) the penicillin-binding component present in the organisms (Rowley, Cooper, Roberts & Lester Smith, 1950) is retained in the cell wall, while rupture in distilled water alone gives inactive cell walls. In order to ascertain the optimum conditions for disruption, a rapid turbidimetric method was developed to estimate the number of bacteria disrupted. It had the advantage of enabling an on-the-spot estimation to be carried out in less than a minute. Thus at least 98% of the organisms could be broken without allowing the suspension to shake or stand more than necessary, so that mechanical and chemical degradation of the cell walls was minimized. The turbidimetric method and some factors affecting rate of rupture of staphylococci are described below.

**METHODS**

*Preparation of bacterial suspensions.* The organism used was a Cowan's type II penicillin-sensitive *Staphylococcus aureus* kindly supplied by Dr Hobbs of the Central Public Health Laboratory, Colindale. After growing on beef tryptic digest agar for 16 hr. at 37° the organisms were harvested in distilled water, passed through a No. 1 porosity sintered glass filter funnel to remove agar particles, and washed 8 times with distilled water.

*Apparatus used for bacterial disruption.* A 'Microid' flask shaker (Messrs Griffin and Tatlock, London) oscillated a maximum load of four shaking tubes (each weighing 100 g. with contents) at a continuously variable frequency of up to 45 cycles/sec., or two tubes up to 58 cycles/sec. The shaking tubes, 140 mm. x 80 mm. and with a row of indentations down each side to ensure
turbulence while shaking, were closed by glass stoppers. The tubes contained 20 ml. bacterial suspension and 20 g. glass 'Ballotini' beads (Messrs Chance Bros., Smethwick, England, 0·1-0·2 mm. diam., grade '12'). The tubes were clamped on the shaker in a horizontal position and shaken vertically with an amplitude of 2·5 cm.

The shaking frequency was determined as follows. A bar magnet was clamped to the shaking arm close to the iron core of a solenoid of approximately 2000 coils which was connected to the Y input of an oscilloscope. A calibrated oscillator connected to the X input was set to a known frequency and the speed of the shaker was continuously tuned to this frequency during shaking by keeping the single Lissajou figure stationary. Without constant adjustment the frequency of the shaker was liable to vary by ±0·5 cycles/sec, and a mains drop of 12 volts caused a frequency drop of 1 cycle/sec. The shaker ran at a different speed if not 'warmed up'. At what appeared to be the same adjustment the frequency differed from day to day. These variations were sufficient to prevent the reproducible rates of breakage necessary to find the optimum conditions, but were completely eliminated by using the tuning system described.

Assay of results. Bacterial dry wt. was determined with a Hilger absorptiometer calibrated in terms of mg. dry wt./ml.

The 'turbidity' of suspensions before and after shaking was measured on 0·25 ml. samples diluted to 10 ml. in distilled water contained in 1 cm. cells in the same instrument with neutral filters. For the present purpose 'turbidity' is taken as the 'Spekker' dial reading, \( = 2 - \log_{10} \text{percentage transmission} \). The percentage of bacteria ruptured was calculated from the relation between the turbidities of a suspension before and after rupture. The relationship is derived below from the rate of drop in turbidity during rupture.

The number of viable organisms present in suspensions before and after shaking was determined by the method of Miles & Misra (1938). Serial dilutions of the suspension were added to 5 ml. broth in capped bottles which were vigorously shaken before removal of the sample for the next dilution. Five drops of each broth dilution were allowed to fall on separate areas of an agar plate which was then tilted and rotated to spread the drops, and the colonies in each of the five groups formed were counted after 48 hr. incubation at 37°. Dilutions were arranged so that the number of colonies per group was 20-50.

The ratio of whole bacteria to cell walls was also observed directly from electron micrographs of washed suspensions.

Composition of shaking media. 'Medium A' contained in 100 ml.: 1 ml. formalin solution, 0·5 g. citric acid, 50 ml. distilled water saturated with octyl alcohol, 0·1 ml. mercaptoacetic acid (thioglycollic acid). These substances were added respectively to reduce enzyme activity, as buffer, to reduce enzyme activity (Meyerhof, 1949) and frothing, and to reduce possible autoxidation. 'Medium B' contained in 100 ml.: 1 ml. formalin solution, 0·5 g. citric acid. Both media were adjusted to pH 6·5 with 10 N-NaOH solution before final adjustment to volume with distilled water.
RESULTS

Changes of turbidity occurring during shaking

The turbidity in distilled water was found to fall non-linearly with time of shaking and reached a nearly level value which was extrapolated to zero time to a value of about 17.5% of the initial turbidity in order to correct for the further slow drop in turbidity on continued shaking (Fig. 1). This value was independent of the frequency of shaking and concentration of bacteria, although the rate at which it was approached depended on these factors.

In ‘Medium A’ and ‘Medium B’ the turbidity fell similarly to level values which were extrapolated to about 33 and 23.5% respectively of initial turbidity. At these levels electron micrographs, staining by Gram’s method, and viable counts where applicable showed that substantially all the bacteria were disrupted. In all experiments the rate of turbidity decrease was less in the presence of formalin than in distilled water alone.

A plot against time of

\[ \log_{10} \left( \frac{\text{turbidity at time} = t}{\text{turbidity at time} = \text{zero}} - 0.175 \right) \]

gave a straight line for distilled water, and substitution of 0.175 by 0.235 for ‘Medium B’ and 0.33 for ‘Medium A’ also gave straight lines (Fig. 2). The values 0.175, 0.235 and 0.33 were chosen as those giving the best average extrapolations and straight lines for a large number of experiments, but from
the range of deviations given in Fig. 2 for two lines it can be seen that they are liable to an error probably of the order of ±0.01 unit. The fact that the values giving the best straight lines corresponded closely with the best extrapolations supports the reasons for the choice of the formula

\[ \log \left( \frac{T_t}{T_0} \right) = \text{constant} \times t \]

which are discussed below.

![Graph showing the relation between log10 [turbidity at time = t (qt)] and time of shaking where \( q_t = 0.175 \), distilled water, \( q_t = 0.235 \), 'Medium B', \( q_t = 0.33 \), 'Medium A', \( q_t = 0.175 \). The deviations shown on two curves represent variations caused by errors of ±0.01 unit in the values for \( q_t \).

Correlation between the percentage of organisms ruptured calculated from turbidity measurements and that observed from viable and electron micrographic counts

It is demonstrated below that in distilled water the percentage of bacteria ruptured, \( P \), after \( t \) minutes shaking should be given by the expression

\[ \frac{P}{100} \left( 1 - \frac{q_t}{q_i} \right) = 1 - \frac{Q}{q_i} \]

where \( q_t \) is the initial turbidity, \( q_t = 0.175 \) and \( Q \) is the observed turbidity at time \( t \). Table 1 shows that the number of ruptured organisms calculated by this means from turbidity measurements agrees well with the number deduced from viable and electron micrographic counts.
Cell rupture in Staph. aureus

Table 1. Correlation between the rates of disruption calculated by means of turbidity measurements and observed from viable and electron micrograph counts. Experiments 1 and 2 performed on separate days but otherwise identical. 2 mg./ml. dry wt. of cells shaken in distilled water at 40 cycles/sec.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Viable counts</th>
<th>Turbidity changes</th>
<th>Experiment 1</th>
<th>% intact cells from</th>
<th>Time (min.)</th>
<th>Electron micrograph counts</th>
<th>Turbidity changes</th>
<th>Experiment 2</th>
<th>% intact cells from</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>43-3</td>
<td>43-2</td>
<td>82</td>
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<td>82</td>
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<tr>
<td>4</td>
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<td>63-1</td>
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<tr>
<td>6</td>
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<td></td>
<td>9</td>
<td>17-4</td>
<td>14-0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Factors affecting rate of rupture

It can be seen from Fig. 2 that in presence of 'Medium B' the rate of rupture is considerably slowed. The rate of rupture is also dependent on frequency of oscillation, so that frequencies less than 30/sec. give a very slow break-up with the apparatus employed (Fig. 3). This rather critical value may be due to the

![Fig. 3](image-url)

Fig. 3. Relation between time required to rupture 90% of Staph. aureus calculated from turbidity measurements, and frequency of shaking in distilled water at 2 mg. dry wt. of cells/ml.

![Fig. 4](image-url)

Fig. 4. Effect of varying Staph. aureus concentration on rate of disruption in distilled water calculated from turbidity measurements. Shaken at 40 cycles/sec. 2 mg. dry wt. cells/ml., ○—○; 5.7 mg./ml., ■—■; 9.2 mg./ml., ●—●.
use of vertical shaking, as little movement of the beads will occur unless the acceleration on the downward throw is greater than that due to gravity.

At bacterial concentrations greater than 2-3 mg./ml. the formation of cell walls ceases to follow a logarithmic law, and the rate of rupture falls off (Fig. 4), but below 2-3 mg./ml. the rate is independent of bacterial concentration. This slowing of rupture is probably due to frothing at the higher concentrations.

DISCUSSION

It can be shown experimentally that the sum of the ‘turbidities’ of suspensions of staphyloccoci at say \(Y/\text{ml.}\) and staphyloccal cell walls at \(Z/\text{ml.}\) is identical with the turbidity of a mixed suspension containing in 1 ml. \(Y\) and \(Z\) of the respective particles, and also that turbidity is directly proportional to concentration for both suspensions. Therefore the turbidity of a staphyloccocal suspension after partial mechanical rupture with glass beads would be expected to consist of the sum of the turbidity due to unchanged cells and the turbidity of the cell walls produced from those bacteria which have been ruptured.

The rate of killing of bacteria by mechanical rupture follows a logarithmic law (King & Alexander, 1948) so that, if complete rupture is the sole cause of killing, the turbidity of a given suspension after \(t\) minutes shaking should equal

\[q_i \cdot e^{-t\lambda} + q_c [1 - e^{-t\lambda}]\]

where \(q_i\) = turbidity of the initial suspension, \(q_c\) = turbidity of the cell walls produced by 100% rupture of that suspension, and \(\lambda\) is a constant.

Thus \(Q\), the observed turbidity at time \(t\),

\[Q_i = q_i \cdot e^{-t\lambda} + q_c [1 - e^{-t\lambda}] = q_i + [q_i - q_c] \cdot e^{-t\lambda}\]

Therefore

\[
\log \left[ \frac{Q - q_c}{q_i} \right] = \log \left[ \frac{q_i - q_c}{q_i} \right] - \frac{t}{\lambda}.
\]

In this equation \(Q\) and \(t\) could be determined experimentally, \(q_i\) was determined before the start of the experiment and \(\frac{q_c}{q_i}\) should be given by the extrapolation of the level values of the turbidities finally reached, as in this case only cell walls were present.

Using these experimental values, a plot of \(\log_{10} \left[ \frac{Q - q_c}{q_i} \right] \) against time where \(\frac{q_c}{q_i}\) was 0.175, 0.235 and 0.33 for distilled water, ‘Medium B’ and ‘Medium A’ respectively, was found to give a straight line. Thus it appeared that the changes in turbidity observed may closely follow the rate of rupture and killing of the organisms. This was confirmed by the close correlation found between the rate of fall of viable counts, rate of rupture observed from electron micrograph counts and the percentage of bacteria broken, \(P\), which can be calculated from the relation

\[Q = q_i \cdot \left( \frac{100 - P}{100} \right) + q_c \cdot \frac{P}{100}, \text{ or } \frac{P}{100} \left[ 1 - \frac{q_c}{q_i} \right] = 1 - \frac{Q}{q_i}.
\]

In practice \(P\) is obtained from the straight line graph relating \(P\) to \(\frac{Q}{q_i}\).
As the rate of fall of viable counts has been found by other workers (King & Alexander, 1948) to be logarithmic, it was assumed above that death only occurs simultaneously with complete rupture in order to deduce a logarithmic fall in turbidity. The fact that the observed results followed the theoretical equations and that viable and electron micrographic counts agreed closely with the production of cell walls calculated by this method confirm this assumption. Thus the staphylococci are able to withstand considerable agitation and only die when completely ruptured. It is not possible to say from this whether partially ruptured organisms are viable, or whether, as appears more likely, the mere splitting of the cell envelope kills the cell and the subsequent complete emptying of the cell contents to leave 'clean' cell walls occurs rapidly.

This correlation between rupture and killing differs from the recent findings of Furness (1952) with *Bacterium coli*, where the total count was always much higher than the viable count. This may possibly be due to the inclusion of partially ruptured organisms in the total count, as in my experience coliforms do not yield 'clean' cell walls on mechanical rupture in as high a proportion as do staphylococci.

The conditions for most rapid rupture obtained from the above data have been described elsewhere (Few et al. 1952).

The slow drop in the turbidity of cell wall suspensions on further shaking, which was more marked at higher speeds, seemed likely to be due to the mechanical disintegration of the cell walls which was noticed in electron micrographs of such suspensions. For this reason it was important not to shake longer or faster than necessary when preparing cell walls which were capable of binding penicillin, as the penicillin-binding component was easily removed by the mechanical grinding provided by the Ballotini beads. Formalin appears to protect the cells to some extent against rupture, and the cell walls prepared in 'Medium A' contain more material and appear considerably thicker in electron micrographs than those from distilled water (Cooper, 1953).

A slight turbidity was observed when Ballotini beads were shaken alone in distilled water, due apparently to small splinters of glass. It did not interfere with the results described above, however, as it never amounted to more than ½% of the turbidities measured, and also much less splintering occurred in the presence of the organisms than in their absence. The very small turbidity contributed by the ultramicroscopic 'lipid particles' of Mitchell & Moyle (1951) also liberated on rupture of the organisms would be included in the term $q_e$.

I wish to express my thanks to London University for a grant for apparatus from the Central Research Fund, and to Dr C. Challice of the National Institute for Medical Research, Mill Hill, London, for electron micrographs.
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(Received 2 March 1953)
The Association of the Penicillin-binding Component of *Staphylococcus aureus* with a Lipid Fraction

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SUMMARY: Radiopenicillin is strongly bound by ultra-microscopic lipid-containing particles liberated on mechanical rupture of *Staphylococcus aureus* cells. The binding resembles that of intact cells in that it is irreversible and only occurs to a limited extent, but differs in that 7–12 times as much penicillin is bound per unit dry weight of material. The supernatant after centrifuging down the lipid particles decreases the titre of added penicillin as indicated by diffusion assay, possibly by a small irreversible inactivation superimposed upon a ‘reversible’ type of binding.

There is a correlation between the distribution of (a) the penicillin-binding component (p.b.c.), (b) $^{35}$S from radiopenicillin pretreated cells, and (c) lipid phosphorus, in the three fractions produced on rupture either in distilled water or in a formaldehyde solution. Rupture in formalin appears to allow the cells walls to retain most of the lipid particles and p.b.c., but only a little extra of the dry weight of the cells. Thus penicillin reacts with a lipid-containing fraction close to the cell wall in intact organisms. At least as much more p.b.c. is liberated on rupture of the cells as was available to the penicillin in the intact cell, but p.b.c. is somewhat unstable after rupture. These data are discussed in the light of evidence in the literature that penicillin may react initially with the osmotic barrier of bacteria.

It was noted several years ago in this laboratory that staphylococcal cell walls prepared by mechanical rupture of cells in distilled water (Cooper, Rowley & Dawson, 1949) were quite white, and that most of the yellow pigment present in the supernatant fraction was sedimented by high-speed centrifugation as a thin orange waxy layer coating the surface of the cell-wall layer. The lipid character of this fraction was suspected from its yellow colour, as nearly all the pigments of staphylococci when extracted by 90% phenol in water were soluble in chloroform. These pigments have been reported to be carotenoid in character (Sobin & Stahly, 1942). Mitchell & Moyle (1951b) confirmed the high proportion of phospholipid in the coloured fraction which sediments more slowly than the cell walls when a mechanically ruptured cell suspension is centrifuged.

It has been reported elsewhere (Rowley, Cooper, Roberts & Lester Smith, 1950) that penicillin-sensitive cells contain a penicillin-binding component (p.b.c.) which appears to be related to the mode of action of penicillin. Cell walls prepared in a formalin mixture were able to bind penicillin (Few, Cooper & Rowley, 1952), whereas cell walls prepared in distilled water were not (Cooper et al. 1949). It was noticed that cell walls which could bind penicillin were yellow and those which could not were quite white and that this colour difference always followed the penicillin-binding capacity. It was, therefore, considered possible that p.b.c. was associated with a lipid fraction of the cells, and the results presented below show that this is the case.
Measurement of penicillin uptake by intact cells and cell walls using radio-penicillin has been previously described (Rowley et al. 1950; Few et al. 1952). Uptake by 'lipid particles' (see below) was measured as described in Table 1. The purity of the radio-penicillin was checked by the methods described by Cooper, Clowes & Rowley (1954).

**Cell preparation.** A penicillin-sensitive *Staphylococcus aureus* was grown on tryptic digest agar in Roux bottles for 16-40 hr., harvested in neutral 0-05M-phosphate solution, filtered through a no. 1 porosity sintered glass funnel to remove agar particles and washed 3 times with distilled water. The organisms were used at once or were freeze dried.

**Disruption of cells.** Cells were disrupted in distilled water or in 'medium A', which consisted of 1 ml. formalin solution, 0-1 ml. mercaptoacetic acid, 0.5 g. citric acid and 50 ml. distilled water saturated with octyl alcohol, made up to 100 ml. with water and adjusted to pH 6-5. Ten ml. of a cell suspension were agitated with 4 g. of glass ballotini beads, no. 12, with a Mickle shaker (Mickle, 1948; Mitchell & Moyle, 1951a). The concentration of cells was up to 25 mg. dry wt./ml. for distilled water, or up to 15 mg./ml. for medium A since the latter did not allow complete formation of typical penicillin-binding cell walls at concentrations higher than this. The rate of rupture was followed by a turbidimetric method (Cooper, 1953). After shaking for 1 hr. 10-15% of the organisms remained intact with distilled water as shaking mixture and 20% with medium A. Little further rupture occurred on continued shaking. In all quantitative analytical work unruptured cells present in the cell-wall preparation were allowed for.

**Separation of fractions after cell rupture.** The suspension of ruptured cells was removed by a pipette, and when quantitative recovery was required the ballotini were washed 5 times with 2 ml. portions of distilled water. The suspension was spun for 15 min. at 2500 g to remove cell walls and intact cells, and the supernatant was re-centrifuged twice at this speed to sediment cell wall debris which was added to the cell wall fraction. The supernatant, still quite turbid, was centrifuged for 30 min. at 20,000 g to sediment the 'lipid particle' fraction, which appeared as a homogeneous orange translucent pellet free from opaque white cell wall debris. No lipid particles were freed in medium A at concentrations below 15 mg. dry wt. bacteria/ml., but the yellow cell walls formed in this case had to be separated from a white heavier layer which appeared to be denatured protein and which contained no P.B.C., or 35S from radio-penicillin pretreated cells. The separation was easily effected with a Pasteur pipette followed by differential centrifuging. The white layer was added to the supernatant fraction for assay purposes.

**Biological penicillin assays** used an agar plate diffusion method with *Sarcina lutea* as test organism, porcelain cylinders of 8 mm. diameter, and an incubation time of 16 hr. at 27° (Welch, 1948). Penicillin solutions containing 0-02 and 0-05 u./ml. gave inhibition zones of 20 and 30 mm. diameter respectively so that the decrease in titre of about 0-01 u./ml. observed in presence of the
supernatant fraction was well outside experimental error. Tenfold replicates were used.

Assay of lipid phosphorus. The procedure followed was similar to that described by Mitchell & Moyle (1951b). The weighed freeze-dried samples (c. 20 mg.) were refluxed for 1 hr. with 2 ml. methanol in 20 ml. tubes with B14 sockets and condensers and the cooled extracts were transferred to fresh tubes. This procedure was repeated twice and the pooled methanolic extracts were evaporated to dryness and kept overnight in vacuo over P2O5. The dried extracts were then re-extracted overnight at room temperature with 3 ml. Na-dried ether, and the ether extracts transferred to fresh tubes; this step was also repeated twice. The pooled extracts were evaporated to dryness and the fatty residues digested with 0·5 ml. conc. HNO3 (AR) + 1 drop of 100 vol. H2O2 until colourless. The total phosphate in these solutions was estimated by the colorimetric method of Fiske & SubbaRow (1925).

RESULTS

Penicillin binding by lipid particles

The lipid particle fraction obtained by high-speed centrifuging of the cell wall-free 'cytoplasmic contents' of the cells after rupture in distilled water was found to bind penicillin strongly (Table 1), the uptake being 10-15 u./g.,

Table 1. Uptake of radiopenicillin on lipid particles before and after addition of 0·2 u. ordinary penicillin/ml.

<table>
<thead>
<tr>
<th>Concentration of penicillin before washing (u./ml.)</th>
<th>Untreated</th>
<th>Pre-treated with ordinary penicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0·1</td>
<td>1410</td>
<td>100</td>
</tr>
<tr>
<td>0·2</td>
<td>1050</td>
<td>100</td>
</tr>
<tr>
<td>0·3</td>
<td>1730</td>
<td>100</td>
</tr>
<tr>
<td>0·4</td>
<td>1960</td>
<td>100</td>
</tr>
</tbody>
</table>

i.e. 7–12 times greater than the original cells. It can be seen that this uptake is independent of penicillin concentration and is completely prevented by pretreatment of the cells with a small amount of non-radioactive penicillin. The uptake by the lipid particles is thus similar to that by intact cells in that it appears to be an irreversible reaction with a component present in limited amount.

The significantly high value of the supernatant radioactivity of the untreated preparation (col. 3, Table 1), compared with that of the penicillin
Penicillin binding and lipids

pre-treated controls (col. 6), suggests that about 20% of the P.B.C.-radio-
penicillin complex present in the lipid particles may be soluble, and indeed it
has been found that less material is precipitated after the second centrifugation
than after the first; thus 5–6% by weight of the bacteria appeared as lipid
particles (Table 2), but only 3–4% was left after one wash. Resuspension in
a larger volume of water (50 ml.) was sufficient to dissolve the lipid particles
completely, leaving no deposit on centrifuging.

The decrease in penicillin titre caused by lipid particles when resuspended
in distilled water, as determined by biological assay, agreed well with the
uptake of radiopenicillin. When the concentration of lipid particles was about
1 mg. dry wt./ml., about 25% of this biologically assayed P.B.C. was still
present in the supernatant after centrifuging for 1 hr. at 20,000 g. A similar
proportion of P.B.C. (about 30% of the total recovered) was present in the
supernatant fraction after rupture (see below). These data indicate that the
amount of P.B.C. present in the lipid particles which will dissolve in 1 ml. will
bind about 0.01 units of penicillin.

Decrease in penicillin titre caused by the supernatant fraction

In order to measure the amount of P.B.C. in the clear supernatant from
high-speed centrifugation after rupture in distilled water, standard penicillin
solutions were equally diluted in the supernatant and in distilled water,
placed at once on the assay plates and incubated. It was found that this
supernatant fraction decreased the titre of the penicillin solutions to an extent
which was dependent on penicillin concentration (Fig. 1). The straight-line
portion could be extrapolated to cut the vertical axis at 0.75 u. penicillin/g. dry
wt. of cytoplasmic contents (c. 0.01 u. penicillin/ml. supernatant). At low
penicillin concentrations only a small proportion of the penicillin which had
been added was still detectable, whereas this proportion was much larger at
higher penicillin concentrations. Fig. 1 suggested that the irreversible and
limited binding by the small amounts of soluble P.B.C. which were expected
to be present was superimposed on a different type of penicillin inactivation.
To see whether this was enzymic, in which case further incubation should
decrease the titre even more, the supernatant fraction was incubated with
penicillin for 6 hr. before adding to the assay cups. As the very small further
decrease in titre then observed was only of the same order as that of a control
in distilled water, it appeared more likely to be due to binding, perhaps by
protein, for example. If such were the case, the binding was reversible, as
estimation of bound radiopenicillin by removing excess radiopenicillin from
the supernatants by an electro-dialysis method (Few, Cooper & Rowley, 1958,
and unpublished results) gave a straight line of very much lower slope. As this
line passed through the origin no P.B.C. was detectable, and the P.B.C. must
therefore be considered to be dialysable. The rate of dialysis of the radiopeni-
cillin was also much slower in the presence of supernatant material than
in its absence, suggesting that the removal of the penicillin ions was being
hindered by a reversible binding.

It is interesting that the reversible binding by supernatant material is very
large compared with the irreversible binding by intact cells (Table 2). No reversible binding of a similar order by the intact cell was found in this present work or by Maass & Johnson (1949a, b) or by Rowley et al. (1950), using

![Penicillin titre decreased](image)

**Fig. 1.** Decrease in penicillin titre caused by the supernatant fraction obtained by centrifuging ruptured suspensions of *Staph. aureus* (20 mg. dry wt./ml.) at 20,000 g for 30 min. The suspension was removed from the ballotini beads without dilution. 'Total penicillin available' represents the amount of penicillin inactivated if the penicillin titre was decreased to zero.

**Table 2.** Distribution of lipid P, P.B.C. and $35S$ (from radiopenicillin pre-treated staphylococci) in the three fractions obtained on mechanical rupture

<table>
<thead>
<tr>
<th></th>
<th>% dry wt</th>
<th>% u./g.</th>
<th>% u./g.</th>
<th>% µmole/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruptured in distilled water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact cells</td>
<td>(100)</td>
<td>20-28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cell walls</td>
<td>(100)</td>
<td>0</td>
<td>0</td>
<td>0-2</td>
</tr>
<tr>
<td>'Lipid particle' fraction</td>
<td>5-6</td>
<td>60-75</td>
<td>10-13-6</td>
<td>19-5</td>
</tr>
<tr>
<td>Supernatants</td>
<td>60-66</td>
<td>60-70</td>
<td>12-5</td>
<td>0-3</td>
</tr>
<tr>
<td>Totals</td>
<td>85-90</td>
<td>90-105</td>
<td>69</td>
<td>70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>% dry wt</th>
<th>% u./g.</th>
<th>% u./g.</th>
<th>% µmole/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruptured in 'medium A'</td>
<td></td>
<td></td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>Intact cells</td>
<td>(100)</td>
<td>2-2</td>
<td>1-5</td>
<td>68</td>
</tr>
<tr>
<td>Cell walls</td>
<td>(100)</td>
<td>4-5-6</td>
<td>2-9</td>
<td>77</td>
</tr>
<tr>
<td>'Lipid particle' fraction</td>
<td>1-2</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Supernatants</td>
<td>60-70</td>
<td>*</td>
<td>12-5</td>
<td>0-3</td>
</tr>
<tr>
<td>Totals</td>
<td>95</td>
<td>60-75</td>
<td>88</td>
<td>87</td>
</tr>
</tbody>
</table>

* No suitable assay
biological and radioactive assay methods. Thus the reversible binding centres
appear to be liberated on rupture. Reversible binding of penicillin G (Klotz,
Urquhart & Weber, 1950) by bovine serum albumin, when expressed as u.
penicillin/g. dry wt., is of a similar order to that of the supernatant fraction
in the present case.

Correlation between distribution of penicillin-binding component from untreated
cells, \(^{35}\)S from radiopenicillin pre-treated cells, and lipid phosphorus

Table 2 shows that P.B.C., \(^{35}\)S and lipid P are practically absent from cell
walls prepared in distilled water, present in the lipid particles to a high con­
centration and in the supernatant fraction to less than or the same amount as
in intact cells. The proportions of \(^{35}\)S closely followed the proportions of
lipid P, and the proportions of P.B.C. are similar, although greater differences
may be expected here since some extra P.B.C. is produced on rupture (see
below) and the moderate stability of P.B.C. when in solution may differ from
that of the lipid particles. A similar correlation was found in medium A, where
the bulk of the P.B.C., \(^{35}\)S and lipid P were retained in the cell wall while only
a small extra proportion of the dry weight was retained.

Differences in rupture products obtained from distilled
water and medium A

In medium A the ‘lipid particle fraction’ was 1–2 % as compared with 5–6 %
in distilled water (Table 2). In fact, the small sediment observed was not at all
similar to the lipid particles from distilled water, being neither orange nor
waxy, but white and opaque and resembling finely divided cell-wall material.
The supernatants from medium A were practically colourless, whereas those
from distilled water were yellow. On the other hand, the cell walls from
medium A were orange, whereas those from distilled water were white.

Effect of lipid solvents

Refluxing for 1 hr. with methanol or pyridine, or standing in 90 % (w/v)
phenol in water removed the bulk of the \(^{35}\)S from radiopenicillin pre-treated
cells. Refluxing dried organisms 3 times with 3 ml. MeOH for 5 min. (under
N₂) destroyed or extracted all the P.B.C. from the cells, together with a con­
siderable amount of lipid material. Unfortunately, whether destruction or
extraction had occurred could not be decided as the extraction products
interfered with the biological assay.

High recovery of penicillin-binding component after rupture in distilled water

Table 2 shows that, while the recoveries of material measured by dry weight,
\(^{35}\)S and lipid P were 85–90 %, the recovery of P.B.C. was always at least 90 %.
This was surprising since cell-free P.B.C. was found to be rather unstable, and
2–2½ hr. had elapsed between the start of rupture and the time of assay. Also,
the uptake by lipid particles represented only that amount of penicillin
remaining bound after one wash in distilled water, and as shown above
P.B.C. is slightly soluble. This suggested that more P.B.C. was liberated on
rupture than was available to the penicillin in the intact cells, an idea which
was supported by the finding that the penicillin \(^{35}\)S bound by lipid particles
was 2-3 times as much per unit dry weight as the amount of \(^{35}\)S recovered in the lipid particle fraction from cells saturated with radiopenicillin before rupture (Table 2, cols. 3 and 5). When 0-3 u. radiopenicillin/ml. was present during cell rupture in distilled water instead of being added afterwards, twice as much radiopenicillin was bound by the lipid particles (i.e. 19-27 u./g. compared with 10-13 u./g.). Presumably the penicillin reacted with the P.B.C. immediately it was formed, thus avoiding any inactivation of the P.B.C.

The high recovery of P.B.C. was confirmed by pre-treating the cells with non-radioactive penicillin and removing the excess by thorough washing. The uptake of radiopenicillin by a sample of the intact cells was then less than 0-08 u./g., yet on rupturing the remainder the uptake by the lipid particles was 4-5 u./g., i.e. about the same as when radiopenicillin was added, and the excess removed, before rupture. The recovery of P.B.C. in the lipid particle fraction alone in this particular experiment, by radioactive assay, was then greater than 400% of that available to the penicillin in the starting material. These results indicate that considerably more P.B.C. is liberated on rupture of the cells than could react with the penicillin before rupture, and that P.B.C. is somewhat unstable during rupture.

**DISCUSSION**

The results presented above show that the component in staphylococci which binds penicillin is associated with a lipid fraction situated close to the cell wall of intact organisms, and suggest that this penicillin-binding component may itself be a lipid. However, this may not be the case, as, calculated on the basis of the lipid phosphorus, the lipid particles would only contain about 10% of material of the composition of lecithin. The lipid particles ('small particle fraction') prepared from a *Staphylococcus aureus* strain by Mitchell & Moyle (1951b) contained components other than lipids, some of which, as these authors pointed out, were very similar in composition to the cell-wall material which did not bind penicillin. They suggested that the small particle fraction was the lipid layer observed cytologically under the cell wall. Their analyses for this fraction were, in fact, consistent with 20-30% fat-soluble material, 30-70% of adhering cell-wall material and a few per cent of substances (e.g. nucleic acids) which were perhaps included from the soluble supernatant fraction, as the particles could not be washed.

Nevertheless, the association of P.B.C. with cell-wall lipid is very interesting in view of the fact that the component responsible for the osmotic properties of the cell, the 'osmotic barrier' (Mitchell, 1949a), must necessarily lie close to the cell surface, and is widely believed to be lipid in character (e.g. see Work & Work, 1948). The fraction associated with P.B.C. has both of these properties. If, therefore, in its initial rapid reaction with the cell the penicillin molecule inactivates some component of the osmotic barrier, then the metabolic defects whose appearance coincides with the addition of penicillin should be closely related to osmotic barrier malfunction. Many such relations appear to exist in the literature, so that it is worth while to consider those surface changes which begin immediately the penicillin is added.
Penicillin binding and lipids

(1) Effects of penicillin on the functions of an osmotic barrier. Gale & Taylor (1947) showed that viability and active amino-acid transport across the osmotic barrier diminishes in parallel fashion in cells growing in presence of penicillin. Hotchkiss (1950), with different bacteria and growth conditions, found that only a little glutamic acid assimilation was prevented by penicillin but that polypeptides were liberated into the medium. This effect was confirmed by Gale & Paine (1951). However, as penicillin inhibits Bacillus subtilis in a medium containing only inorganic N (Hunter & Baker, 1949), the cell's disorganization is not limited to amino-acid uptake. A contrasting phenomenon, the effect of osmotic barrier destruction on penicillin uptake, is that loss of penicillin-binding capacity by intact cells and lipid particles proceeds simultaneously with cytolysis by a lipid-soluble substance such as phenol, whereas an anionic and a cationic detergent had no effect over the cytolytic range (Cooper, 1954).

(2) Effect of penicillin on surface properties of the cell. The cell surface is apparently synthesized in situ and is, therefore, either concerned in its own manufacture or is synthesized by the layer immediately underlying it, i.e. the lipid 'cytoplasmic membrane'. (There is some cytological evidence of cell wall synthesis by the cytoplasmic membrane, e.g. Bisset, 1950.) Thus it is relevant to this discussion that penicillin added to growing cultures immediately slows the formation of a polyhydroxy phosphoric ester (XSP) which comprises a large proportion of the cell wall of most penicillin-sensitive, but not resistant, organisms (Mitchell & Moyle, 1950). Since the cell surface increases, because the cell also swells, it follows that either the cell wall becomes very much more elastic, perhaps due to loss of XSP, or that a very considerable amount of other cell wall components are produced to make good the deficiency of XSP. It may be a reflexion of this change that the suspension simultaneously loses its stability (Mitchell & Moyle, 1951a) and that there occurs a progressive drop in surface charge which starts very soon after addition of penicillin (McQuillen, 1950–1). The very small number of penicillin molecules attached (Rowley et al. 1950) would themselves contribute a negligible amount to the charge on the bacterial surface. Streptomycin causes an opposite type of effect (McQuillen, 1951) in that there is an immediate change in surface charge on addition of this compound to resting cells, corresponding to a reversible adsorption of it, but there is no further effect on growing cells. The effect of penicillin in this case is, therefore, not common to all antibiotics, as is, for instance, the formation of 'giant forms'.

Another surface change reported is that a penicillin-resistant brucella organism lost the power to synthesize its surface capsule only when in presence of penicillin (Braun, Kraft, Mead & Goodlow, 1952). It is interesting that these surface changes presumably do not apply to the whole surface, as growth in penicillin does not affect the adsorption of phage on a sensitive staphylococcus (Elford, 1948).

(3) Changes in properties of the strain on adaptation or selection to penicillin resistance or sensitivity. When penicillinase production is not involved, changes in penicillin sensitivity are simultaneously accompanied by changes in many
properties. Among these are changes which appear to be related to osmotic or surface properties of the cells. For instance, highly resistant staphylococci lose ability to grow in 6.5 % NaCl (Bellamy & Klimek, 1948; Klimek, Cavallito & Bailey, 1948), and resistant brucella mutants lose their smooth character (Braun et al. 1952). Gale (1947) found that an increase of, say, tenfold in penicillin resistance resulted in the glutamic acid assimilation system requiring tenfold the amount of penicillin to inhibit it, and the more resistant organisms were less able to assimilate glutamate (Gale, 1947). When relating changes in antibiotic sensitivities to the osmotic barrier, however, it must be remembered that any general change in cell permeability is likely to alter drug sensitivity.

In conclusion, the very small number of penicillin molecules bound by sensitive bacteria makes it seem probable that a very limited number of reactions, perhaps only one, may be governed by p.b.c. If this is so, the very diverse nature of the effects produced by penicillin in different organisms suggests that this reaction which is initially disorganized by addition of penicillin itself controls many diverse systems. The osmotic barrier in bacteria plays a vital part in the economy of the cell and it is shown above that there are many suggestions that the initial lesion caused by penicillin lies in the osmotic barrier. It is worth consideration, therefore, that the effects of penicillin in disorganizing, for example, nucleic acid metabolism (Mitchell, 1949b; Gale & Rodwell, 1948; Park & Johnson, 1949) may be due to the inability of the cell to assimilate, synthesize or retain some substance essential for such metabolism because of faulty functioning of the osmotic barrier.

I wish to thank Sir Alexander Fleming, F.R.S., for his continued interest in this work, and Dr D. Rowley for the help afforded by many useful discussions. Acknowledgements are due to the Central Research Fund, London University, for a grant for apparatus, and to Dr E. Lester Smith, of Glaxo Laboratories, for a generous gift of radiopenicillin G.

This paper is the second in the series 'The site of action of penicillin'.

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Penicillin binding and lipids


(Received 17 August 1953)
THE SITE OF ACTION OF PENICILLIN

III. EFFECT OF SURFACE-ACTIVE SUBSTANCES ON PENICILLIN UPTAKE

BY STAPHYLOCOCCUS AUREUS

by

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During attempts to extract that component of penicillin sensitive staphylococci which is capable of binding the drug it was observed that 30 mg/ml aqueous phenol solutions were capable of preventing penicillin uptake. As the penicillin binding component (PBC) is associated with a lipid fraction close to the cell wall in intact staphylococci, the effects of detergent substances on such a fraction is of interest in view of the disorganising effects of detergents on that fraction of the cell responsible for retaining cell solutes, which must certainly lie close to the cell surface and is widely believed to have lipoidal properties (see for example WORK AND WORK).

METHODS

The method for determination of radiopenicillin uptake was described by Rowley et al., and growth conditions, preparation of "lipid particles" by mechanical rupture of the cells and measurement of radiopenicillin uptake by these particles were described by Cooper. The effect of detergents was determined by addition of detergent to the concentration indicated, after which cells were centrifuged once and resuspended in water. Samples were taken for viable counts and radiopenicillin was added to the suspensions. The supernatant after centrifuging was assayed for total inorganic phosphate by the method of Fiske and Subbarow and the amount of phosphate liberated was taken as an indication of the cytolyis which had occurred. To determine its effect on penicillin uptake by lipid particles, phenol was added directly to the suspension of lipid particles in the "cytoplasmic fluid" remaining after rupture of the cells in distilled water and removal of cell walls by centrifuging. Radiopenicillin was added, and the uptake of radiopenicillin was measured as described.

Viable counts were made by the method of Miles and Misra. The purity of the radiopenicillin was ascertained by the method described by Cooper, Clowes and Rowley. Phenol used was AR crystalline and dioctyl sodium sulphosuccinate (Aerosol OT), cetyl trimethylammonium bromide (CTAB) and Tween 80 were the commercial products.

RESULTS

Effect of phenol on penicillin uptake

In the experiments shown in Fig. 1 no cells were killed up to 3 mg phenol/ml but all cells were dead at 6 mg/ml. It can be seen that "cytolysis" (i.e. liberation of inorganic phosphate) appears to start only when all the cells are killed, although in fact the viable count is measured after a much longer time of contact with phenol than cytolyis or uptake, as there is presumably phenol still attached to the cells during the incubation period. Thus the process of death proceeds to completion while cytolyis is measured.

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after only one hour. Cytolysis and prevention of penicillin uptake proceed at a measurable rate over concentration ranges which are identical (i.e. 5–20 mg/ml). As cytolysis increases, the uptake by intact cells and by lipid particles decreases (phenol added after rupture). Penicillin $^{35}$S, once attached, is not removed by all concentrations up to 50 mg/ml phenol. Attempts to recover PBC from 30 mg/ml phenol extracts by freeze-drying were not successful. (PBC was estimated by addition of the extracts to penicillin solutions, which were then compared with known standards by a plate assay procedure. No inactivation of penicillin was found to occur.)

![Graph](image1)

Fig. 1. Effect of different phenol concentrations on penicillin uptake by lipid particles (O—O) and by intact staphylococci (●—●), and on P$^{43}$ liberation (○—○). Cells at 3 mg/ml were contacted with phenol at $18^\circ$ for 1 h, centrifuged, the supernatant was removed for P$^{43}$ assay, the cells were resuspended in distilled water for viable counts, and radiopenicillin was added. Cells were centrifuged after a further 30 min and assayed for radioactive uptake.

Phenol, in concentrations which completely prevented penicillin uptake, had no effect on the naked eye appearance of lipid particles when sedimented, i.e. they still appeared as an orange homogeneous translucent pellet of the same size. This suggests that, although phenol disorganises the osmotic barrier, this is not due to gross solubilisation of lipid material. Cells treated with phenol before rupture gave poor yields of whitish lipid particles, not well defined.

The inhibiting effect of 30 mg/ml phenol on penicillin uptake by intact cells appeared to be complete within the 10 minutes required for assay, and up to at least 45 mg dry wt. cells/ml. There was some indication that the reaction proceeded more slowly around 10 mg phenol/ml. MITCHELL AND McQUILLEN\(^{10}\) reported that cytolysis by phenol at 10 mg/ml required about one hour for completion.

Effect of dioctyl sodium sulphosuccinate (Aerosol OT) on penicillin uptake

The changes in Aerosol OT (Fig. 2) differ from those in phenol in that death and cytolysis start at lower concentrations and proceed simultaneously, although 90% death

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again occurs at much lower concentrations than 90% cytolysis. The logarithm of the viable counts falls off linearly with concentration. In contrast with phenol, even when 99.9% of the cells are killed and cytolysis is perhaps 90% complete, penicillin uptake is decreased only by 10–20%. No $^{35}$S from radiopenicillin-pretreated cells is removed at 2 mg/ml Aerosol OT.

![Fig. 2. Effect of different Aerosol OT concentrations on penicillin uptake by intact staphylococci (---), $\text{PO}_4^{3-}$ liberation (●●●), viability (○○○) and $^{35}$S remaining on radiopenicillin-pretreated cells (□□□). Treated as for phenol, except that 5 mg dry wt. cells/ml were contacted with Aerosol for 2 h.](image)

**Effect of Tween 80 (polyoxyethylene sorbitan mono-oleate) on penicillin uptake**

Penicillin uptake was not affected by Tween 80 up to 20 mg/ml but was reduced from about 2.2 u/g dry wt. at 0–20 mg Tween 80/ml to 1.75 u/g at 50 mg/ml. This slight drop in uptake at 50 mg/ml may be due to small quantities of oleic acid present as an impurity, functioning as an anionic detergent in a similar manner to Aerosol OT.

**Effect of cetyl trimethylammonium bromide (CTAB) on penicillin uptake**

In increasing CTAB concentrations penicillin uptake increases gradually until the cells agglutinate and are all killed, when very much more penicillin becomes attached (Fig. 3). Penicillin $^{35}$S attached before contact with CTAB is little affected. Of the high radiopenicillin uptake at high CTAB concentrations (Fig. 4), some is prevented by addition of small amounts of non-radioactive penicillin after the CTAB but before the radiopenicillin, and is therefore irreversibly attached. This irreversible uptake is roughly independent of concentration. The remainder of the uptake is not prevented by ordinary penicillin and is proportional to penicillin concentration, and is presumably reversibly bound. This may well be due to radiopenicillin which has not been sufficiently washed out, as the agglutinated suspensions are very difficult to wash by centrifuging.

It can be calculated from Salton's data that the concentration of detergent added will be $2–3 \times$ that of the true concentration of CTAB present in the supernatant at References p. 438.
equilibrium due to the high binding of detergent by the cells. Bearing this in mind, there is a coincidence between the added CTAB concentrations where penicillin uptake increases sharply and the calculated supernatant CTAB concentration where (a) the surface properties of the cells change sharply (seen from agglutination, and found by electrophoresis by McQuillen), (b) saturation of the cells with CTAB becomes

Fig. 3. Effect of different CTAB concentrations on penicillin uptake on intact staphylococci (●—●) viability (○—○), and 35S on radiopenicillin pretreated cells (□—□). Treated as for phenol.

Fig. 4. Uptake of radiopenicillin on CTAB-treated staphylococci. Of 12 tubes containing cells at 3 mg/ml, 8 were treated with CTAB added to 2 mg/ml for 30 min, all 12 were centrifuged, and the cells resuspended in distilled water. Ordinary crystalline penicillin (0.2 μ/ml) was added to 4 of the CTAB-treated tubes, all tubes were centrifuged again after 20 min and resuspended in distilled water. Radiopenicillin (0.5, 1.0, 1.5 and 2.0 μ/ml respectively) was then added to each of the tubes in the three sets of four and the cells were re-centrifuged after 30 min for assay of 35S uptake.

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maximal$^5$ and (c) micelles begin to form$^{12,18}$. This coincidence suggests the probability that the high irreversible penicillin uptake in CTAB is due to a strongly adsorbed salt formation between penicillin and those CTAB molecules which may be secondarily adsorbed as micelles onto the primary monolayer of adsorbed CTAB.

**DISCUSSION**

The four types of detergent substances examined above each appear to cause different effects upon penicillin uptake by staphylococcal cells. Tween 80, electrically neutral and lipid insoluble, is not very cytolytic or bactericidal and so it is perhaps not surprising that penicillin uptake is not affected. Phenol, also a neutral detergent yet lipid soluble, causes a marked drop in penicillin uptake by intact cells and by lipid particles which exactly corresponds with cytolysis, while Aerosol OT, negatively charged and lipid insoluble in the usual sense, causes little effect on penicillin uptake over the cytolytic and bactericidal concentrations. CTAB, on the other hand, causes a marked increase in uptake of penicillin$^{35S}$ but not until concentrations have been reached where cytolysis may be presumed to be complete (Salton$^5$). Over the cytolytic range the uptake is increased less markedly, but no decrease in PBC similar to that with phenol was noticed.

As PBC was not recovered from phenol extracts, and no penicillin$^{35S}$ once attached was removed by any detergent, it appears likely that PBC is not removed from the cells by phenol but rather is blocked in some way. Thus although phenol and the ionic detergents all disorganise the osmotic barrier, the former prevents penicillin reacting with PBC during cytolysis of the cells while the latter do not. Some evidence has been presented$^2$ to suggest that PBC was actually associated with the osmotic barrier in that (a) it was in a lipid fraction close to the cell wall, and (b) some metabolic disturbances which occurred soon after contact with penicillin were cell wall phenomena. The coincidence of the prevention by phenol of penicillin uptake and of the destruction by phenol of the osmotic barrier affords further evidence in support of this idea. It also suggests a fundamental difference between the reactions of phenol and ionised detergents with the osmotic barrier, which may perhaps be expected from the ability of phenol to dissolve completely, and not just at the interface, in lipid bodies.

However, since it is not yet possible to isolate a moiety clearly recognisable as "the osmotic barrier", as is possible with the cell wall for instance, an association of penicillin mode of action with the osmotic barrier can only be assumed from indirect evidence. Several pieces of indirect evidence are available, and it remains to be seen whether sufficient further information on the properties of the osmotic barrier is forthcoming to make it seem more certain that such an association exists.

**ACKNOWLEDGEMENTS**

I wish to thank Sir Alexander Fleming, F.R.S., for his continued encouragement, and Drs. D. Rowley and A. V. Few for helpful discussion and criticism.

**SUMMARY**

Phenol prevented penicillin uptake by *Staphylococcus aureus* whole cells and "lipid particles" at the same concentrations in which cytolysis and cell death occurred. Tween 80 had little effect on penicillin uptake. Aerosol OT caused only a small decrease in penicillin uptake over the concent-

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tation range in which cell death and cytolysis were maximal, but CTAB caused an increase in penicillin uptake which was especially marked above the agglutination level. No penicillin $^{35}S$ was removed from the cells by detergents once the penicillin had been attached. The relationship of these findings with the hypothesis that penicillin reacts initially with the osmotic barrier in Staphylococcus aureus is discussed.

Résumé


Zusammenfassung


REFERENCES


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The Site of Action of Penicillin: some Properties of the Penicillin-binding Component of *Staphylococcus aureus*

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SUMMARY: The penicillin-binding component (PBC) of *Staphylococcus aureus* is rapidly inactivated by acid but is more stable at neutral or alkaline pH values. At 2° various preparations lost 25-50% activity overnight at the optimum pH. Cell-free preparations of PBC (penicillin-binding cell walls or 'lipid particles') showed an initial increase in the amount of PBC available to penicillin. PBC was heat-labile, being completely destroyed by 5 min. at 50°. The final amount of penicillin bound did not vary over the range pH 4.4-7.6, but the rate of binding was somewhat greater at the lower end of this range. No separation of PBC from lipid particles was achieved by several mild techniques. Organic solvents which did not remove lipid material from intact cells also failed to affect PBC; more drastic solvent procedures which were effective in removing lipid appeared to destroy PBC. Intact cells or lipid particles from a penicillin-resistant yeast did not bind penicillin.

The most likely interpretation of the experimental finding (Rowley, Cooper, Roberts & Lester Smith, 1950) that penicillin-sensitive cells were able to bind a small definite amount of penicillin in a specific and irreversible manner was that they contained a small amount of a chemical entity which was termed the penicillin-binding component (PBC). It was thought that PBC was likely to be the initial site of action of penicillin, although direct proof of this could not be afforded. PBC might exist in the cell as an easily removable co-factor or it might be an integral part of a large molecule, and its isolation would then involve the difficult task of separating a very small amount of material from a large amount of similarly constituted impurities. Alternatively, it is theoretically possible that PBC cannot exist separately, but is rather a steric effect produced by the conjunction of two large molecules between which the penicillin happens to fit. Any attempt to separate the two large molecules would then lead to the disappearance of PBC. Nevertheless, it seemed worth while to attempt the preparation of PBC in a soluble form from which chemical purification could begin. The results given below describe some properties of PBC and the lipid-containing particulate fraction in which it is concentrated (Cooper, 1954), and illustrate some of the difficulties encountered in obtaining PBC in soluble form.

METHODS

Uptake of radiopenicillin by intact cells was measured by the method described by Rowley *et al.* (1950), and the preparation of, and measurement of uptake by, penicillin-binding cell walls were as described by Few, Cooper & Rowley (1952). Methods of growing the staphylococcus used, preparation of 'lipid particles' and measurement of penicillin-binding by lipid particles by
Penicillin-binding component

use of radiopenicillin and by bioassay were described by Cooper (1954). The radiopenicillin purity was checked by methods described by Cooper, Clowes & Rowley (1954).

RESULTS

Stability of penicillin-binding component

It was important to know to which experimental conditions PBC could be submitted without undue loss. The results illustrated in Fig. 1 show that PBC could be kept at neutral or alkaline pH values for several hours at room temperature even in the cell-free state, but was rapidly and irreversibly inactivated by acid. About 25% of the PBC was lost from intact cells when kept overnight at 2°C at the optimum pH value (pH 6). In one sample of lipid particles stored overnight at 2°C in the presence of cell cytoplasm about 50% of the initial PBC was lost. The curious suggestion (in Fig. 1) that the penicillin-binding ability of cell walls prepared in formalin (Few et al. 1952) had increased slightly during the course of the experiment was confirmed in several experiments; it always occurred during the 1–2 hr. following the completion of the formalin cell-wall preparation and then fell fairly rapidly (Fig. 2). A similar

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**Fig. 1.** Effect of pH value on stability of PBC in 0.1 M-phosphate + 0.1 M-acetate. Assays of the initial activity were made separately in each case. ○—○, intact cells, left for 16 hr. at various pH values and 2°C, washed 3 times and adjusted to pH 7 before addition of radiopenicillin. ●—●, cell walls prepared in formalin mixture left for 45 min. at various pH values and 2°C, washed twice and adjusted to pH 7 before addition of radiopenicillin. ○—○, unwashed lipid particles, left for 90 min. at various pH values and 2°C, neutralized by addition of penicillin in an equal vol. 2 M-phosphate buffer at pH 7.5; binding determined biologically by cup-plate assay of residual unbound penicillin.

**Fig. 2.** Effect of time of standing in distilled water at 18°C on the penicillin-binding capacity of cell walls prepared in formalin mixture. Radiopenicillin was added at various times, and the suspensions then kept in the cold until the end of the experiment, when all were centrifuged and washed together for 35S assay.
increase in available PBC but without subsequent rapid loss also occurred with lipid particles which had been separated from the cytoplasmic supernatant; it proceeded at about the same rate at 0° as at 37° (Tables 1 and 2). This, with the lack of inhibition of the effect by formalin pretreatment, suggested that the increase was not enzymic. Approximately twice as much PBC is liberated on cell rupture as is available to the penicillin in the intact cell (Cooper, 1954). The final uptake reached in Table 1 corresponds closely with the uptake by lipid particles when inactivation of PBC during rupture is minimized by shaking in the presence of radiopenicillin. The ability of the lipid particles to regain their penicillin-binding capacity on standing suggests that this inactivation may be reversible.

Table 1. Stability of PBC in unwashed lipid particle suspensions in distilled water at 0° and 37°

At the beginning of the experiment about 2 hr. had elapsed from start of cell rupture. Penicillin was added (0-07 unit/ml.) at various times and the suspensions then kept at 0° until the end of the experiment. The residual penicillin concentrations were determined by bioassay.

<table>
<thead>
<tr>
<th>Time of observation (hr.)</th>
<th>Amount of penicillin bound by particle suspension (unit/ml.)</th>
<th>Penicillin bound by lipid particles (unit/g. dry wt. particles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0° 27°</td>
<td>0° 27°</td>
</tr>
<tr>
<td>0</td>
<td>0-008 0-008</td>
<td>6-4 6-4</td>
</tr>
<tr>
<td>1/2</td>
<td>0-031 0-041</td>
<td>25 33</td>
</tr>
<tr>
<td>2</td>
<td>0-036 0-028</td>
<td>29 22</td>
</tr>
<tr>
<td>3</td>
<td>0-040 0-032</td>
<td>32 25</td>
</tr>
</tbody>
</table>

Table 2. Effect of temperature on stability of PBC

Experimental method as in Table 1.

<table>
<thead>
<tr>
<th>Amount of penicillin bound by lipid particle suspension (unit/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initially</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>0-081</td>
</tr>
</tbody>
</table>

The apparent difference in rates of loss of PBC from penicillin-binding cell walls and from lipid particles is probably due to the several washes of the former involved in the assay of bound penicillin. Thus PBC may become progressively more easily washed away from cell walls on standing.

PBC was unstable at higher temperatures, none being detectable after 5 min. at 50° (Table 2). Freeze-drying had no effect on the PBC content of intact cells, lipid particles or formalin-prepared cell walls. The PBC-radiopenicillin complex remained attached to intact cells in aqueous suspension very much longer than PBC itself was detectable.
Penicillin-binding component

Effect of pH value on penicillin uptake

Fig. 3 shows that the final amount of penicillin bound by resting cells (20 min. total contact) was only a little affected between pH 4-4 and 7-6. Very similar curves were obtained when the penicillin solutions were adjusted to the various pH values for the same period of time and neutralized before addition of cells, showing that the higher uptakes at pH 3 and 4-4 were entirely due to radio-active decomposition products of penicillin. The lowest pH value at which instability of penicillin is small over the course of the experiment is about pH 4-4 (Brodersen, 1947), increasing greatly at lower pH values. However, although the final amount of uptake of radiopenicillin is independent of pH, the rate at which this value is attained is decreased with increase in pH value (Fig. 4). The effect of pH value on the rate of penicillin uptake is of interest in view of reports (Abraham & Duthie, 1946; Eagle, Levy & Fleischman, 1952) which indicated that penicillin is considerably more active at pH 6 than at pH 7-5. Since the minimal inhibitory titre may be the result of a balance between the rate of inactivation of PBC by penicillin and the rate of PBC resynthesis (Maass & Johnson, 1949b), any increase in the rate of inactivation of PBC caused by pH changes should result in this balance being struck at lower titres, since the rates of penicillin-binding at pH 5-5 and 7-3 are roughly proportional to penicillin concentration (Fig. 5). It can be calculated from Figs. 4 and 5 that penicillin at 0-025 unit/ml. and pH 7-5 should inactivate PBC as fast as at 0-017 unit/ml. and pH 6-0. The difference between these theoretical minimal inhibition concentrations is rather less than the two- or fourfold differences observed by Abraham & Duthie and by Eagle and colleagues over this pH range, so that changes in penicillin binding-rate are probably not the only effects of pH value on the lethal process caused by penicillin.

Some properties of the lipid particles

The lipid particle suspension could not be fractionated by any of several mild methods. Electrophoresis at pH 9-0, 7-4 and 6-0 gave single ascending and descending boundaries which became less distinct with time and rapidly moved at different rates towards the anode, suggesting a polydisperse system bearing a negative charge. PBC remained in the fast-moving fraction. The pH value of maximum precipitation (iso-electric point?) was between pH 3 and 4, so that fractional precipitation at lower pH values would involve too much loss of PBC to be practicable. Differential centrifugation of lipid particles over the range 8000–16,000 g produced no changes in the PBC or lipid P assays. A fairly constant amount of lipid particle was soluble in each ml. of distilled water but the saturated solution was too dilute to produce a visible boundary in the electrophoresis apparatus. Both lipid particles and PBC were completely precipitated by calcium ions and by freezing and thawing, but were unaltered in appearance by boiling. A preparation of Clostridium welchii α-toxin (containing the lecithinase C) in 0-01 M-CaCl₂ or 1 mg. crystalline ribonuclease or trypsin per ml. at 37° for 30 min. had no effect on PBC or on the lipid particles.
Fig. 3. Effect of pH value on the uptake of radiopenicillin on intact cells suspended at 5 mg. dry wt./ml. in 0.1 M-phosphate + 0.1 M-acetate. Three samples of cells were adjusted to pH values of 3.0, 4.4, 7.6 respectively, each sample was divided into five portions and differing concentrations of radiopenicillin was added to each portion. After 15 min. at 18° the cells were rapidly centrifuged and washed 3 times in water for 35S assay.

Fig. 4. Effect of pH on rate of penicillin uptake. Six samples of cells were adjusted to various pH values in 0.1 M-phosphate + 0.1 M-acetate and radiopenicillin was added to 0.02 μg/ml at zero time. Half of each sample was rapidly centrifuged and washed after 30 min. at 18° (○—○), and the remainder after 60 min. (●—●), for 35S assay.

Fig. 5. Effect of penicillin concentration on rate of uptake at pH 5.53 (○—○) and pH 7.35 (●—●). Cells were suspended in 0.1 M-phosphate + 0.1 M-acetate, the suspensions were adjusted to pH 5.53 or pH 7.35 and added to the prepared radiopenicillin dilutions. After 30 min. at 18° the samples were centrifuged rapidly and washed for 35S assay.
At room temperature addition of ethanol, 5% ethanol, ethyl ether, chloroform, acetone, 5% phosphate (pH 9-0), 9% urea or 10% NaCl solutions to freeze-dried cells did not remove PBC; nor did adjustment of a thick cell suspension to pH 9, 10, 11 or 12 followed by neutralization of the separated extracts. Under these conditions, the organic solvents mentioned were also ineffective in removing lipid. Trichloroacetic acid (10%, w/v), anhydrous formic acid, anhydrous pyridine, cyclohexylamine, 90% phenol, hot methanol and anhydrous diethylene glycol rendered the cells incapable of irreversibly binding radiopenicillin, but no PBC (by bioassay; or by electrodialysis assay method, Few, Cooper & Rowley, 1958) was detected in the residues from extracts made with trichloroacetic or formic acids, cyclohexylamine, pyridine or 90% phenol (dialysed or freeze-dried to remove solvent), with diethylene glycol (dialysed or ethanol-precipitated) or with hot methanol (distilled off in vacuo).

Treatment with 90% phenol, followed by cold acetone and ether, did not extract any $^{35}$S from cells grown in radiopenicillin and washed thoroughly, although all the lipid and lipid P was extracted. This suggests that the penicillin did not react with the lipid fraction of the lipid particles, although any solvent procedures which succeeded in extracting cell lipid (90% phenol, pyridine, hot methanol, cyclohexylamine) also succeeded in destroying PBC.

**Uptake of penicillin by yeast**

The findings of Maass & Johnson (1949a) that penicillin was not bound by yeast have been confirmed in this laboratory with a strain of baker's yeast. Daniel & Johnson (1954) showed that cell-free extracts of *Saccharomyces carlsbergensis* bound penicillin in an apparently specific manner, but radiopenicillin uptake experiments in the present work did not reveal any irreversible binding of penicillin by the lipid particles of the baker's yeast used (i.e. less than 0.1 unit penicillin at 0.4 unit/ml.). Perhaps yeast species contain PBC within the cell and sheltered from penicillin, rather than at or near the surface as in *Staphylococcus aureus*. It is interesting that briefly heating the supernatant from centrifuged ruptured staphylococcal cell suspensions appears to destroy the 'reversible' binding by cell contents (Daniel & Johnson, 1954; Cooper, 1954).

**DISCUSSION**

It can be seen that considerable difficulties are involved in the separation of PBC in a chemically definable form. All attempts to prepare a soluble extract were unsuccessful except for a simple saturated solution of lipid particles which was rather dilute to handle chemically or electrophoretically. Attempts to concentrate this solution produced coagulation on freeze-drying or inactivation by heat. PBC is very unstable to heat and acid, and cannot be kept long even at low temperature and at the optimum pH. Accordingly, extraction procedures must not be used which require prolonged standing in the presence of water, e.g. dialysis or solvent precipitation.
The so-called ‘lipid particles’ contain a large amount of protein (Mitchell & Moyle, 1951) as well as about 20% of lipid material and PBC, and their behaviour as outlined above indicates their chemical constitution to include a sparingly soluble lipo-protein complex in which the fatty material is strongly bound. PBC also appears to be strongly bound, but it may be more closely connected with the protein part. This is perhaps to be expected if penicillin specifically interferes with some enzymic activity, although binding is not affected by trypsin. Daniel & Johnson (1954) also found that their PBC was not affected by trypsin. The effect of trypsin may thus be a useful method of distinguishing the specific binding by PBC from the non-specific binding present in supernatants from ruptured cell suspensions (Cooper, 1954).

I should like to thank Dr D. Rowley for his helpful criticism, advice and support. I wish to acknowledge a grant from London University Central Research Fund for apparatus.

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SUMMARY: To see whether the initial damage caused by penicillin involved the osmotic barrier, certain characters of *Staphylococcus aureus* were examined at intervals during the first 2 hr. after addition of penicillin to growing cultures. In the 30 min. following addition of penicillin the cell appeared to expose nearly all its reserve penicillin-binding component (PBC), the penicillin uptake being double that which occurred without growth and the reserve PBC disappearing. The amount of PBC in the cell and its rate of exposure to penicillin in excess of normal synthesis (rate of turnover?) were both very small. Between 30 and 60 min. the uptake of Na, Mg, K, P and increase of total dry matter ceased abruptly. Continued increase in dry wt., while P, Na, Mg and K decreased slightly, resulted in the penicillin-treated cells becoming relatively deficient in these elements. Synthesis of lipid phosphorus and some, but not all, large molecule phosphates still continued, and Fe and Co uptake were not affected. After 60-80 min. water entered and solutes began to leave the cell, and synthesis of large molecule P ceased. The primary site of action of penicillin is probably not concerned with gross assimilation of Na, K, Mg, P, Fe, Co, or any substance contributing more than 10% of the dry wt., or with gross synthesis of lipid P. It may, however, involve a reaction turning over 3000 times while the cell mass is doubled.

To describe fully the mode of action of a drug one must account for all the changes which take place from the moment of first contact between cell and drug. Although this is clearly impossible to do in detail, some such attempt is necessary as it is very difficult to pin-point any particular defect as being the cause of death. The specific nature of the penicillin molecule and the small amounts bound by cells suggest that the changes caused by the drug are sequences of events branching from perhaps only one small and specific injury. Each event plays its part in a sequence which culminates in the inability of most of the population to divide, and the relative time of appearance of abnormalities is important.

It was shown elsewhere (Cooper, 1954) that penicillin is firmly bound in a specific manner by a lipid-containing fraction near the cell wall of a sensitive staphylococcus. Believing that the penicillin-binding component (PBC) of sensitive cells (Rowley, Cooper, Roberts & Lester Smith, 1950) is likely to be the initial site of the bactericidal action of penicillin when acting at its minimal concentration, it was suggested that its presence in this fraction might indicate that the first effect of penicillin is to disorganize some function specific to the cell wall or to the osmotic barrier. To obtain further information on this possibility, penicillin was added to growing cultures of sensitive staphylococci,
Early effects of penicillin

and the following characters were examined very soon after addition of penicillin: (a) the 'phosphate-impermeable volume' (the space enclosed by the osmotic barrier to phosphate) and the volume of the centrifuged cell pad; (b) the dry wt. of the 'lipid particle' fraction, and the phosphate of lipid and other fractions; (c) ability of the cell to take up and retain the elements it requires from the medium. (These abilities may or may not be related to osmotic barrier function for elements other than P.)

The observations described are therefore attempts to obtain some idea of the sequence of defects occurring during the first hour or two after addition of penicillin, paying particular attention to possible cell wall or osmotic barrier activities which might be the site of action of penicillin.

METHODS

The organism was the penicillin-sensitive (0-02 unit/ml.) Staphylococcus aureus used previously (Cooper, 1954), which was capable in March 1954 of reproducibly binding penicillin in the resting state to 0-90 ± 0-1 unit/g. dry wt. It is interesting that the penicillin-binding capacity of this strain has steadily dropped from 2-4 units/g. in 1949 without concurrent change in penicillin sensitivity or colonial appearance, but the average cell size has increased, so that the number of penicillin molecules bound/cell has not changed. The number of cells/g. dry wt. calculated from viable counts was $8 \times 10^{11}$, but calculations from the average cell diameter (1-3 $\mu$) and specific volume (c. 5 ml./g.) give about $3-5 \times 10^{12}$ cells/g. dry wt. This figure, itself liable to considerable error, has been used to calculate that about 300 molecules of penicillin are bound per cell in the resting state. The difference from alternative figures for other strains (Maass & Johnson, 1949 a; Eagle, 1954) is probably largely accounted for by the difficulty in assessing the number of cells/g. dry wt., as uptakes in units penicillin/g. dry wt. were often similar.

Growth conditions. Most of the experiments involved taking serial samples after adding penicillin to a culture growing under standard conditions, with simultaneous samples from a control culture. Tryptic digest agar was inoculated with young cultures, the cells were harvested aseptically after 16 hr. at 36°, washed twice with sterile distilled water and the dry matter of the suspension was determined turbidimetrically by reference to a calibration curve. Samples of such suspensions were added to 100 ml. warm broth contained in 1 l. long-necked B34 round-bottomed flasks plugged with cotton-wool, so that the final concentrations were 0-2, 1-0 or 2-5 mg./ml. These were shaken with c. 10 mg. of Silicone antifoam A (Hopkins and Williams Ltd.) at about 5 cyc./sec. on a Microid shaker (Griffin and Tatlock Ltd.) at 36°. These conditions gave a mean generation time of about 1 hr., which with this organism allowed a good separation in time of many of the metabolic defects caused by penicillin.

Penicillin was generally added 1 hr. after inoculation, when the cells were just entering the logarithmic phase, and 10 ml. samples, taken at intervals, were rapidly cooled in ice and washed once in distilled water, so that the final
sample contained less than 1/2000 ml. medium. Penicillin was usually used in the lowest concentration at which penicillin binding is complete within a minute or two at 37°, i.e. 0-1 unit/ml. This concentration is also the lowest at which the rate of bactericidal action is maximal (Eagle, 1951), and at which the production of fresh viable units is immediately stopped (Parker & Marsh, 1946; and present work). High concentrations may give rise to other less specific effects of penicillin.

**Measurement of PBC.** Assay of PBC by use of radiopenicillin and the preparation of radiopenicillin were as described by Rowley *et al.* (1950); radiopenicillin purity was checked by the methods of Cooper, Clowes & Rowley (1954).

**Cell volume.** Cell-pad volume was determined by centrifuging 0-25 ml. of cell suspension containing about 0-05 ml. of cells for 30 min. at 3500 *g* in tubes of 2-5 mm. internal diameter. A mark was made at the top of the cell mass, and the wt. of mercury occupying the volume in the cleaned and dried tubes determined. The phosphate impermeable volume was determined as that volume not penetrated by $^{32}$PO$_4$, in presence of excess phosphate and carried out rapidly at 0° so that negligible exchange of $^3$P could occur between cells and medium. For this, 0-25 ml. of the same cell suspension was added in the cold to 0-1 ml. of 2M-phosphate at pH 7 containing 0-4 μc. $^{32}$P/ml. After mixing, the suspensions were at once centrifuged for 5 min. at 5000 *g* in a refrigerated angle centrifuge using tubes 0-6 mm. internal diameter, and two samples of 0-1 ml. of the supernatant were rapidly removed for $^{32}$P assay. Samples for ‘cell-pad’ and ‘phosphate impermeable’ volumes were thus centrifuged separately at different rates and ionic strengths, and are not comparable with each other. The average cell-pad volume (6-8 ml./g.) is high compared with certain other staphylococci (3-47 ml./g., Gale, 1947; 3-61 ml./g., Mitchell, 1953), but the organism used here was rather larger than average. The data in Fig. 1 suggests that the specific volumes of cells may not be absolute, but probably depend on growth conditions and speeds and ionic strengths obtaining during centrifuging.

**Dry matter.** Samples of 10-20 mg. cells in weighed 20 ml. tubes were dried at 110° for 16 hr., cooled over P$_2$O$_5$ in vacuo, stoppered and re-weighed.

**Phosphorus determinations.** Inorganic phosphate was determined by the method of Fiske & SubbaRow (1925), organic phosphates being first combusted with ‘Analytical Reagent’ perchloric acid. Cell suspensions were treated with an equal volume of 6% phenol solution for 20 min. at room temperature to obtain the phenol-soluble phosphates, comprising inorganic (PhI) and organic (PhO) fractions which corresponded quantitatively in normal cells of this staphylococcus with the trichloroacetic acid-soluble (AI and AO) and butanol-soluble phosphates prepared by Mitchell’s method (1953). The PhI in the supernatant was determined directly without combustion and the PhO was determined as (total phenol-soluble P–PhI). In the whole cells or fractions $^{32}$P was assayed by counting samples dried on planchettes, making due allowance for radioactive decay. To determine $^{32}$P in the PhO, PhI was first precipitated as NH$_4$MgPO$_4$ by addition to 5 ml. samples of 0-1 ml. 20 N-ammonia
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and 0.1 ml. of 5% (w/v) MgCl₂·6H₂O, leaving PhO in solution after 16 hr. at 4°C. Contaminating PhO in the washed precipitate was less than 5% PhP. PhP³²P was determined as total phenol-soluble³²P minus PhO³²P. The relative specific activities ("R value", or ratio of observed value of³²P/³¹P to that value expected at complete equilibration) were expressed as described by Mitchell & Moyle (1953). The total P and³²P were determined in the residue after phenol treatment, which was taken as representing the large molecule phosphate.

Determination of other elements. Iron was determined as³⁰Fe and by the thiocyanate colorimetric method. The samples were combusted to 0.1 ml. with HClO₄ and 0.1 ml. 10% (v/v) HNO₃ and 0.5 ml. water were added; the samples were cooled, mixed with 3 ml. acetone and 0.5 ml. of 20% (w/v) NH₄NO₃, and made up to 5 ml. and the Fe assayed after 30 min. on a Hilger absorptiometer using a blue filter. Sodium, potassium, magnesium and calcium were determined with the Hilger 'Uvispek' photoelectric spectrophotometer using a flame photometer attachment and the quartz prism. Standards contained approximately the same proportions of Na, K, Ca, Mg and PO₄ as found in the samples, and the experimental conditions shown in Table 1 were used (compare Kapuscinski, Moss, Zak & Boyle (1952) for Mg and Ca). Co was determined as³⁰Co, and by the arc spectrograph.

Viable counts were made by the method of Miles & Misra (1938).

Table 1. Experimental conditions used for flame photometer assays

<table>
<thead>
<tr>
<th>Element</th>
<th>Wave length (Å)</th>
<th>Minimum standard (µmole/ml.)</th>
<th>Min. conc. determined (µmole/ml.)</th>
<th>Slit width (mm.)</th>
<th>Pressure H₂ (lb./sq.in.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>5890</td>
<td>0.2</td>
<td>0.02</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>K</td>
<td>7790</td>
<td>0.5</td>
<td>0.05</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>Mg</td>
<td>3820</td>
<td>2</td>
<td>0.2</td>
<td>0.8</td>
<td>2</td>
</tr>
<tr>
<td>Ca</td>
<td>5540</td>
<td>1</td>
<td>0.1</td>
<td>0.6</td>
<td>1</td>
</tr>
</tbody>
</table>

RESULTS

Changes in cell volume during growth in presence of penicillin

The well-known swelling of organisms in the presence of lethal amounts of penicillin is unaccompanied by increase in cell mass (Fig. 1), and therefore differs from the formation of 'giant forms' in sub-inhibitory titres (e.g. Fleming, Voureka, Kramer & Hughes, 1950) where increase in cell mass occurs but division is inhibited. Fig. 1 shows a progressive decrease in cell-pad and phosphate-impermeable volumes of normal cells during growth under the conditions used here, and penicillin had very little effect on these volumes up to about 60 min. after its addition. However, soon after the dry matter ceased to increase there was a marked increase in the phosphate-impermeable volume of the cells, and a somewhat smaller increase in the cell-pad volume. The changes in cell-pad volume may reflect changes in cell-packing (perhaps due to changes in mutual adhesiveness) rather than in the volume of individual cells, but the similar nature of the changes in phosphate-impermeable volume
suggests that this is not so. The important point is that growth in the presence of penicillin causes no rapid collapse in the cell-pad and phosphate-impermeable volumes similar to that produced by addition of butanol or trichloroacetic acid (Mitchell, 1953). About 60 min. after the addition of penicillin, the apparent increase of these volumes without corresponding increase in dry matter suggests that the cells rapidly took up a considerable amount of water. This, with the slow liberation of PO₄ and decrease of dry matter which commences soon after, suggests that an increase in the permeability of the osmotic barrier occurs at this stage. Alternatively, the cell wall may become more elastic due to loss of PGP, the polyglycerophosphate complex described by Mitchell & Moyle (1951b), or some internal breakdown of large molecules may cause a rise in the internal osmotic pressure. Therefore, at most, breakdown of the osmotic barrier's passive properties has only just begun by 60–100 min. after addition of penicillin.

Fig. 1. Changes in cell-pad and phosphate-impermeable volumes of Staphylococcus aureus on growth in the presence of penicillin. The washed cells (equivalent to 1 mg. dry wt./ml.) were added to warm shaken broth and penicillin (to 0.1 unit/ml.) was added 60 min. later. Samples were taken at intervals. The phosphate-impermeable and cell-pad volumes are not comparable with each other, as different methods of centrifuging were employed. Normal culture, — O ; penicillin-treated culture, —••—••.

Changes in cell dry weight

In agreement with the observations of Parker & Marsh (1946), 0.1 unit penicillin/ml. was found to be the lowest concentration at which production of fresh viable units ceased immediately on addition of penicillin. In spite of this, seven separate experiments, including those of Figs. 1 and 4, showed that dry matter increased in identical manner to that of the control for 54–60 min.
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after addition of penicillin, when the increase suddenly ceased. (The fourth 'penicillin' point was low in the dry wt. curve of the experiment shown by Fig. 2.) This was similar to the experience of Park (1951), where increase in turbidity, dry weight, nitrogen, phosphorus or nucleic acid was almost normal for 45 min. after addition of penicillin. This time was very much the same for many experiments, with the exception of one (Table 2), although the control cultures took from 57 to 800 min. to double their weight from the time of adding penicillin. The abruptness of the cessation of the uptake of materials comprising the bulk of the dry matter of the cell, namely carbon and nitrogen compounds, indicates that they all cease to enter the cell at about the same time. The dry matter remained unchanged for a further 60 min. and then gradually decreased.

Table 2. The lack of effect of penicillin on iron uptake

Washed freshly harvested *Staphylococcus aureus* cells were added equivalent to 1 mg. dry wt./ml. to warm broth containing 0.22 μmole Fe/ml and 0.0025 μC. ⁵⁹Fe/ml at zero time. Penicillin (to 0.1 unit/ml) was added 60 min. later. Samples of 10 ml were taken at intervals, cooled and the cells washed twice with broth and once with distilled water, so that ⁵⁹Fe uptake represents irreversibly bound iron. No difference was found when the broth washing was omitted.

<table>
<thead>
<tr>
<th>Period after inoculation (min.)</th>
<th>Growth (mg. bacterial dry wt./ml. culture)</th>
<th>Bacterial Fe bound (μmole/ml. culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + penicillin</td>
<td>Control + penicillin</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>1.33</td>
<td>0.0070</td>
</tr>
<tr>
<td>61</td>
<td>2.32</td>
<td>0.0175</td>
</tr>
<tr>
<td>80</td>
<td>2.68</td>
<td>0.0245</td>
</tr>
<tr>
<td>103</td>
<td>3.68</td>
<td>0.0400</td>
</tr>
<tr>
<td>122</td>
<td>3.86</td>
<td>0.0446</td>
</tr>
<tr>
<td>142</td>
<td>4.12</td>
<td>0.0526</td>
</tr>
<tr>
<td>162</td>
<td>4.15</td>
<td>0.0549</td>
</tr>
<tr>
<td>180</td>
<td>4.29</td>
<td>0.0637</td>
</tr>
</tbody>
</table>

Changes in elementary composition

If PBC were itself the 'shuttle' by which a medium constituent is transported into the cell, the entry of this constituent should be rapidly stopped by penicillin above 0.1 unit/ml. If so, the constituent is not glucose (or oxygen) as the oxidation of this substance is unaffected by growth for at least 1 hr. in penicillin (Gale & Paine, 1951) and penicillin is still active anaerobically (see below). It is not an organic substance other than glucose, as penicillin is equally effective on media from which these are missing (Hunter & Baker, 1949; Grelet, 1949). This leaves only the inorganic constituents. Although P uptake is known to be an active function of the osmotic barrier in the staphylococcus used by Mitchell (1953), little is known of the uptake mechanism for K, Na, or Mg. Even so, it would be interesting to see whether the uptake of these metals is affected by penicillin.

Fig. 2 and Table 4 show that the increase of total dry wt. and the uptake of P, K, Na and Mg stopped quite abruptly at intervals after addition of
penicillin which did not quite coincide, so that when dry wt. increase had ceased the cells were relatively somewhat deficient in these elements. Usually some loss of these elements started soon after dry wt. ceased to increase.

![Graphs of Dry matter, Phosphorus, Magnesium, Potassium, and Sodium changes over time.](image)

**Fig. 2. Changes in elementary composition of Staphylococcus aureus growing in presence of penicillin.** Conditions as for Fig. 1. Values refer to quantities of element in cells/ml. culture.

**Table 3. Effect of 0-1 unit penicillin/ml. on $^{60}$Co uptake**

Uptake is expressed as $\mu$C. bacterial $^{60}$Co/l. culture, as accurate assays of Co in cells and medium were not available. Cells contained about 5 $\mu$g. Co/g. dry wt., medium about 0-5 $\mu$g. Co/ml. To each of four 100 ml. shake-cultures 4 $\mu$C. of $^{60}$Co were added 65 min. after addition of cells equivalent to 1 mg./dry wt./ml. Penicillin was added to the three test cultures, 60, 75 and 115 min. after inoculation.

<table>
<thead>
<tr>
<th>Period after inoculation (min.)</th>
<th>Control</th>
<th>Penicillin added at 60 min.</th>
<th>Penicillin added at 75 min.</th>
<th>Penicillin added at 115 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>0-326</td>
<td>0-428</td>
<td>0-320</td>
<td>0-326</td>
</tr>
<tr>
<td>107</td>
<td>0-376</td>
<td>0-508</td>
<td>0-388</td>
<td>0-376</td>
</tr>
<tr>
<td>131</td>
<td>0-356</td>
<td>0-608</td>
<td>0-380</td>
<td>0-424</td>
</tr>
<tr>
<td>156</td>
<td>0-400</td>
<td>0-522</td>
<td>0-348</td>
<td>0-408</td>
</tr>
<tr>
<td>185</td>
<td>0-396</td>
<td>0-444</td>
<td>—</td>
<td>0-308</td>
</tr>
<tr>
<td>208</td>
<td>0-326</td>
<td>0-416</td>
<td>0-292</td>
<td>0-282</td>
</tr>
</tbody>
</table>

'Uptake' of iron was not affected by penicillin, and cobalt 'uptake' was not inhibited but was sometimes stimulated slightly (Tables 2 and 3). The 'uptake' of Fe and of Co measured here differ from the uptakes of P, K, Na and Mg in
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that they include any equilibration between cell and medium. Ca, rather surprisingly, was present in the washed cells in amounts too small to measure accurately by the method used.

Table 4 also shows the mean elementary composition of the staphylococcal cells during logarithmic growth. The values for P and K were fairly constant over the growth period in the control cells, but the Na content rose to a peak during most active growth and fell towards the end of the logarithmic phase. The Fe and Mg content increased by approximately 70% of the initial value. Allowing for incomplete packing, the cells probably occupy about 5 ml/g of the cell-pellet volume of 7.5 ml/g. It is interesting that the cells contain more P, K, Fe and Co per unit volume than the medium, but less Na, Mg and Ca (unless some is lost in the washing process).

Changes in phosphate distribution

Fig. 2 shows that the net gain of PO₄ from the medium was not affected by penicillin until just before the increase in dry matter ceased. Fig. 3 shows that the accumulation of P in the PhI and PhO stopped about the same time as, or earlier than, the assimilation of total P. In no experiment was there any sign of the PhO (nucleotide?) fraction increasing above that of control. However, net synthesis of the large molecule P (polyglycerophosphate complex (PGP) + deoxyribonucleic acid (DNA) + ribonucleic acid (RNA) + lipid phosphate) continued at the expense of the PhO, but at a slower rate. This suggests that one or more component has ceased to be formed, possibly PGP (Mitchell & Moyle, 1950) as no accumulation of nucleotides suggesting inhibition of nucleic acid synthesis was found. Very little P was lost from the cells until about 90 min. after uptake ceased. Fig. 3 suggests that the cessation of P uptake is due to an interference with the transport mechanism, as otherwise a considerable and lasting accumulation of P in the PhI would be expected when uptake ceased. The alternative possibility, that PO₄ continues to enter the cell's inorganic phosphate pool but in a loosely bound form which was completely washed out between harvesting and assay, is unlikely as Fig. 3 shows that there is still a considerable pool of PhI and PhO phosphates which are not washed out.

Comparison of the rates of entry of ³²P-labelled phosphate into the various fractions (expressed as relative specific activities, or 'R' values, in Table 5) shows that the specific activity of the total P stayed constant at the value occurring as the time uptake ceased, so that there was no exchange between cell and medium after this time. Thus, the active uptake mechanism did not continue simultaneously with an equivalent loss from say, PGP, where P is not known definitely to enter via the inorganic pool. Nevertheless, there were marked rearrangements in the internal distribution of P. The R value of the PhI rose more rapidly than the control initially, then dropped to very low values, while those of the PhO and large molecule P were practically constant for several hours. Mitchell & Moyle (1958) said that it is not known whether the lipid phosphate and PGP are derived from the phosphate of the medium (ml) or the shuttle compound which transports the phosphate across the
Table 4. Elementary composition of Staphylococcus aureus during logarithmic growth

Samples were taken between 60 and 200 min. after addition of 1 mg. dry wt. washed cells/ml. warm broth. Two values in a space indicate the results from two identical experiments, one of which is shown in Fig. 2.

<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>K</th>
<th>Mg</th>
<th>Na</th>
<th>Ca (approx.)</th>
<th>Fe</th>
<th>Co (approx.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmole/g. dry wt. in control (mean 60–200 min.)</td>
<td>1094, 1131</td>
<td>722, 605</td>
<td>200, 218</td>
<td>114, 164</td>
<td>20</td>
<td>8.3</td>
<td>0.1</td>
</tr>
<tr>
<td>μmole/g. dry wt. in control at time of stoppage of dry-matter accumulation in penicillin-treated culture</td>
<td>1132, 1189</td>
<td>677, 633</td>
<td>—, 228</td>
<td>—</td>
<td>153, 178</td>
<td>—</td>
<td>8.3</td>
</tr>
<tr>
<td>μmole/g. dry wt. in penicillin-treated culture, at time of stoppage of dry-matter accumulation</td>
<td>990, 1087</td>
<td>632, 520</td>
<td>—, 173</td>
<td>90, 134</td>
<td>—</td>
<td>8.3</td>
<td>—</td>
</tr>
<tr>
<td>Interval in min. between adding penicillin and cessation of uptake (± 5 min.)*</td>
<td>38, 50</td>
<td>37, 47</td>
<td>—, 45</td>
<td>24, 32</td>
<td>—</td>
<td>&gt; 160</td>
<td>—</td>
</tr>
<tr>
<td>Atoms/cell (approx.) taking 3.5 × 10¹² cells/g.</td>
<td>3 × 10⁸</td>
<td>1.1 × 10⁸</td>
<td>6 × 10⁷</td>
<td>3 × 10⁷</td>
<td>3 × 10⁸</td>
<td>2 × 10⁶</td>
<td>3 × 10⁶</td>
</tr>
<tr>
<td>μmole/ml. wet cells taking 5 ml/g. (see text)</td>
<td>200</td>
<td>120</td>
<td>40</td>
<td>23</td>
<td>4</td>
<td>1.5</td>
<td>0.017</td>
</tr>
<tr>
<td>μmole/ml. medium</td>
<td>10.5</td>
<td>13.7</td>
<td>124</td>
<td>100</td>
<td>70</td>
<td>0.24</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* The times for inhibition of increase of dry matter were 54 and 57 min.

Table 5. The effect of penicillin on the relative specific activities (R) and on total P in various phosphate fractions

Penicillin (0.1 unit/ml) added 6 min. after inoculation of Staphylococcus aureus equivalent to 0.2 mg. dry wt./ml. warm broth containing 32P. Paired values in the body of the table represent R; μmole bacterial P/ml. culture.

<table>
<thead>
<tr>
<th>PhI</th>
<th>PhO</th>
<th>PG + DNA + RNA + LP</th>
<th>Total P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hr.)</td>
<td>Control</td>
<td>+ penicillin</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>0.09</td>
<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>1</td>
<td>0.09</td>
<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>3</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>5</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
</tr>
</tbody>
</table>


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osmotic barrier without turnover, or from AI with turnover. The lack of exchange of $^32P$ between cell and medium after net uptake was stopped by penicillin is evidence that either (a) significant PGP synthesis directly from mI has also ceased by the time the P transport has stopped, or (b) all P enters via the one transport mechanism, and P for PGP comes via AI with turnover, i.e., reversibly. The lack of effect of penicillin on the P uptake until 50-60 min.

Fig. 3. Changes in the distribution of phosphate between the various fractions of *Staphylococcus aureus* on growth in presence of penicillin. Cells equivalent to 0.2 mg. dry wt./ml. were added to warm shaken broth and penicillin to 0.1 unit/ml. was added 95 min. later. (○—○) = PhI, (•—•) = PhO, (® — ®) = 'large molecule phosphate', (© — ©) = total phosphate. In each case the upper curve is the control.

after the addition of penicillin suggests that the P for PGP (comprising nearly 40 % of the total cell P, Mitchell & Moyle, 1951 b) cannot be derived directly from the medium, and at the same time cease to enter PGP much before the total P uptake ceases. This means that the statement of Mitchell & Moyle (1950) that the PGP content falls very rapidly after addition of penicillin is also evidence for (b) above. The changes in specific activity shown in Table 5
suggest that the synthesis of some component(s) of PGP + DNA + RNA + LP, presumably PGP, is inhibited within 60 min. by penicillin, but the reverse loss back into the PhI is still allowed. Table 6 shows that synthesis of total lipid P continued after accumulation of dry matter had ceased. Growth in 0.1 unit penicillin/ml. for 1 hr. did not affect the proportion by weight of the 'lipid particle' fraction sedimenting between 5000 and 12,000 g.

Table 6. Effect of penicillin on lipid phosphorus of growing Staphylococcus aureus cells

Fifty ml. of overnight culture were added to 300 ml. of warm broth in each of 14 Roux bottles, and penicillin (0.06 unit/ml.) was added to 7 bottles 0.66 hr. after inoculation. The contents of one control and one penicillin-treated bottle were harvested at different times during incubation at 36°, washed twice and assayed for lipid P.

<table>
<thead>
<tr>
<th>Time after inoculation (hr.)</th>
<th>Viable count orgs./ml. culture x 10^8</th>
<th>Dry wt. cells μg./ml. culture</th>
<th>Bacterial lipid P μmol PO_4/l. culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>2.2</td>
<td>10</td>
<td>0.83</td>
</tr>
<tr>
<td>1.9</td>
<td>8.8</td>
<td>48.7</td>
<td>1.32</td>
</tr>
<tr>
<td>2.8</td>
<td>16</td>
<td>68.4</td>
<td>1.71</td>
</tr>
<tr>
<td>3.9</td>
<td>38</td>
<td>87.7</td>
<td>2.02</td>
</tr>
<tr>
<td>5.9</td>
<td>62</td>
<td>135</td>
<td>2.84</td>
</tr>
<tr>
<td>6.9</td>
<td>78</td>
<td>186</td>
<td>3.35</td>
</tr>
</tbody>
</table>

Sensitivity of Staphylococcus aureus to penicillin under anaerobic growth conditions

Three drops of broth containing 5, 300 and 15,000 viable cells respectively were placed on each of a duplicate series of agar plates containing 0.2, 0.1, 0.05, 0.025, 0.012, 0.06, 0.08 and 0 unit penicillin/ml. One series was incubated aerobically and the other anaerobically in a McIntosh and Fildes jar at 37°. Although growth was slower and the final size of colony was smaller with anaerobic growth, there was no difference in the number of colonies after 16 and 64 hr. which appeared at each concentration whether grown with or without air. Thus the initial site of penicillin action cannot be concerned with the metabolism of oxygen as such.

Increase of bound penicillin during growth

Provided that no growth occurs in the presence of penicillin, the penicillin-binding capacity of cells is the same whether they are harvested during the logarithmic phase of growth (Fig. 4) or during the stationary phase, or washed with distilled water and suspended in neutral buffer, namely 0.9 ± 0.1 unit/g. dry wt. However, when the cells were grown in the presence of penicillin the uptake progressively increased to approximately double after about 30 min. Previous experiments (Cooper, 1954) showed that resting cells contained a reserve of
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PBC, liberated by rupture, which is approximately equal to the PBC available to penicillin in the intact cell. Thus such cells actually contain twice as much PBC as they appear to in the intact state. From Fig. 4, allowing for this reserve PBC, the total rate of synthesis of PBC in the control culture is about 2·5 units/l. of culture/100 min., whereas the rate of exposure of PBC during the first 30 min. after addition of penicillin is about 10·2 units/l. of culture/100 min. Therefore, either the rate of synthesis is greatly stimulated by penicillin, or else the 50% of the total PBC which is held in reserve is rapidly mobilized and exposed to penicillin, while synthesis continues at the normal rate. The latter view is rendered more likely by the finding that, when cells were grown for 60 min. in presence of 0·1 unit non-radioactive penicillin/ml.,

![Graph](image_url)

Fig. 4. Penicillin binding by Staphylococcus aureus in the growing phase. Cells added equivalent to 2·5 mg. dry wt./ml. (O—O) = control culture, samples (10 ml.) of the culture taken at intervals were mixed with cold radiopenicillin to give 0·1 unit penicillin/ml. Growth was stopped within 1 min. by cooling. (■—■) = penicillin-treated culture, radiopenicillin added to the culture to 0·1 unit/ml at 60 min. Growth was allowed to proceed in presence of penicillin, samples being taken at intervals and rapidly cooled. Both sets of samples were then washed before 35S assay and measurement of dry wt. cells/ml.

washed three times with distilled water and ruptured, the radiopenicillin-binding capacity of the lipid particles was decreased from 12 units/g. (control) to 1·8 units/g. Contact of the intact cells with 0·1 unit non-radioactive penicillin/ml. without growth only decreased the uptake to 6 units/g. Thus, at least 70% of the reserve PBC had been exposed to penicillin during growth, the remaining small uptake by the lipid particles possibly being due to some PBC synthesized during this period of growth and not yet exposed to penicillin. The reserve PBC can be rapidly exposed by mechanical rupture at 2° in presence of 1% formalin when enzymic synthesis would be much slowed, so that exposure without synthesis seems quite possible. Some synthesis of PBC may continue for a time after exposure of the reserve PBC is largely complete.
(Fig. 4). Eagle (1954) was unable to find any significant effect of the metabolic state of bacteria on their ability to bind penicillin, but the increase in uptake on growth found by Cooper & Rowley (1949) and by Few, Cooper & Rowley (1952), was also observed by Maass & Johnson (1949b).

**DISCUSSION**

So far from the osmotic barrier of Gram-positive bacteria being merely a semi-permeable dialyzing membrane which retains large but not small molecules, it is impermeable to phosphate (Mitchell, 1953) while allowing free entry of lysine, (Gale, 1947). The cell is, therefore, obliged to assimilate phosphate by transport mechanisms requiring energy. Little is yet known of the bacterial uptake mechanisms for other elements or water. It may not be sufficient, however, to assume that all positive ions enter merely by diffusion as 'gegenions', as certain authors have invoked active mechanisms for K⁺ transport into erythrocytes (e.g. Ponder, 1950; Harris & Maizels, 1952). Also sheep kidney cortex mitochondria may assimilate Mg⁺⁺, PO₄³⁻, K⁺ and Na⁺ by processes which require energy (e.g. Bartley & Davies, 1954).

*Effects of penicillin on properties of the osmotic barrier.* The association of PBC with cell-wall lipoprotein suggests that penicillin may initially disorganize some function of the osmotic barrier (Cooper, 1954). The glutamate assimilation is unaffected until about 60 min. after addition of penicillin (Gale & Taylor, 1947) when it is suddenly very seriously impaired, but it is not at present certain whether, as seemed likely earlier, the uptake of glutamate is in fact by active transport mechanisms (Gale, 1953). The present work shows that at least one active mechanism, namely phosphate assimilation, abruptly ceases to function at about the same time, even if its inactivation is not irreversible. It might also be inferred from the sudden stoppage of increase of dry matter soon after, while the cell can still probably synthesize peptides or proteins, deoxyribonucleic acid and ribonucleotides from internal resources (Gale & Taylor, 1947; Hotchkiss, 1950; Gale & Paine, 1951; Gros & Macheboeuf, 1953; Mitchell & Moyle, 1951a; present work), that the replication of the bulk of cell material (carbon and nitrogen compounds) is blocked at the stage of taking into the cell. The time course of the impairment of K⁺, Na⁺ and Mg⁺⁺ uptake closely resembles that of dry matter and P, but it remains to be seen whether these metals enter by active or passive transport mechanisms. If passive, then uptake cessation is due to prevention of synthesis or uptake of their several receptors (not peptides, certain large molecule phosphates, or glucose oxidation products), and is not a direct result of impairment of osmotic barrier function. Nevertheless, whether or not these changes represent a general impairment of the active functions of the osmotic barrier, it is evident that they take some time to appear, so that they probably represent secondary effects of penicillin. Certain properties such as retention of solutes, and possibly exclusion of water, are also not affected until somewhat later, so that breakage of the osmotic barrier is not an early effect of penicillin either. This is possibly reflected in the unaffected synthesis of lipid phosphates.
Early effects of penicillin

Later secondary effects of penicillin. Meanwhile, respiration, fermentation and oxidation of glucose, and lysine uptake (Gale & Taylor, 1947; Gros & Macheboeuf, 1953) continue with peptide and nucleic acid synthesis, although the nucleoide-oxidizing ability is considerably impaired by the time that turbidity reaches its peak. In view of the probable drop in internal pH value due to depletion of alkali metals while respiration continues and the increase in osmotic permeability, it is in fact surprising that more enzyme deficiencies have not been noticed, particularly those involving Mg and P. As the glutamate assimilation system appears to require Mg or Mn for its energy supply (Gale, 1949), the deficiency of Mg may be the cause of its impaired function. Also K+ stimulates uptake of glutamic acid by *Staphylococcus aureus* (Davies, Folkes, Gale & Bigger, 1953).

Deficiency of certain elements may also account for another effect of penicillin. The cellular acid-soluble ribonucleotides increase very markedly on growth with penicillin, while RNA remains steady (Park, 1952a-c, 0.1 unit penicillin/ml.; Mitchell & Moyle, 1951a, 1 μg. penicillin/ml.) and this could be interpreted as a block in RNA synthesis at the stage of nucleotide polymerization. Synthesis of DNA is apparently unaffected. The present work shows that the use of 3% phenol to liberate the small molecular components of the cell did not reveal any accumulation of soluble nucleotides after addition of penicillin. On the contrary, large molecule P synthesis continued at the expense of the pool of phenol-soluble organic phosphorus. It is, therefore, suggested that penicillin allows ribonucleotides to enter RNA normally for a time, but the cell’s elementary deficiencies somehow weaken this RNA synthesis, so that the more recently added ribonucleotides are removed by the rather drastic treatment with trichloroacetic acid. The RNA isolated from Gram-positive cells contained about 5% firmly bound Mg (Henry & Stacey, 1946) and Mg is essential in considerable amounts for Gram-positive cells (Webb, 1951). Absence of Mg decreases the rate of RNA synthesis to one-tenth in *Escherichia coli* (Abelson & Aldous, 1950), but it is not known at present what role if any metallic elements play in nucleic acid structure. It is interesting that the giant forms induced by deficiency of Mg (Webb, 1951) are similar to those formed in sub-lethal penicillin concentrations (Fleming, Vourekka, Kramer & Hughes, 1950), although it is true that other substances besides penicillin can induce giant forms.

The nature of the reaction controlled by PBC. The exact nature of this reaction is still not known; none of the characters examined was damaged very soon after adding 0.1 unit penicillin/ml. It is tempting to speculate that the increased bound penicillin caused only by growth in the presence of penicillin is the result of the forced substitution of the normal substrate of PBC by penicillin. Thus the obscured half of the total PBC may be progressively diverted by penicillin from duties which involve it in a routine cycle, and the rate of PBC release (above that due to its normal synthesis) would represent the normal rate of turnover of PBC when metabolizing its substrate. If so, it
can be calculated from this rate and from the rate of increase of dry matter (Fig. 4) that the 'free' PBC turns over about ten times in order to double the cell mass. Each cell will bind 300 molecules of penicillin, and if 1 molecule of penicillin displaces 1 molecule of PBC substrate, then in order to produce two cells from one, PBC would metabolize 3000 molecules of substrate—the amount of substrate required by one cell. Several assumptions are involved here, but even if this rough calculation is ten times too low, then a quantitative change in a reaction of such a small size would still require a very specific analytical method to detect it.

On the bases of (1) the effectiveness of penicillin in the absence of all organic matter other than glucose, and in the absence of air, and (2) the likelihood that above 0.1 unit penicillin/ml. the use of PBC is completely stopped either immediately penicillin is added (as all free PBC is bound within 2–3 min. or at least by the time of the earliest observed abnormality—i.e. block in Na uptake, 20–30 min.), it is possible to say what are not functions of PBC, namely: gross assimilation of oxygen, Na, Mg, K, P, Fe, Co, or material contributing largely to the dry matter, including all organic substances; synthesis of the major part of lipid phosphates and the lipid particle fraction. The very small turnover rate of PBC deduced above would also exclude uptake of Ca, and synthesis of the major part of fractions present in large amount such as PGP, DNA, RNA, cell wall material and proteins, although synthesis of a minor component of these fractions would be possible but difficult to detect. It is also possible that PBC is the shuttle mechanism for the intake of a trace element required in amounts similar to those of cobalt, although if this be so the shuttle is not actuated by thermal agitation as is that for phosphate (Mitchell, 1953), since no turnover of PBC occurs in the resting state.

In conclusion, it may be said that although penicillin is bound by a component very close to the osmotic barrier and most of the earliest large-scale changes have some connexion with active properties of the cell wall, yet these changes do not begin with this organism until at least 20–30 min. after addition of penicillin under conditions where the initial lesion should be evident at once. It remains to be seen what is the function of the system initially affected by penicillin, and how this small defect causes damage to certain cell wall activities while other metabolic activities continue.

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Early effects of penicillin


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A full description of the "mode of action" of a drug should include all the changes which the drug brings about from its first contact with the susceptible cell. Perhaps from the difficult nature of this task, implying as it does a full knowledge of what is normal also, and from the obvious importance of the initial step in the series of changes, most interest is usually attached to the nature of this initial damage. However, the bacterial cell must contain very many individual metabolic systems, and without some guide the chances of stumbling upon the very reaction which is first damaged seem rather remote. Thus, although chemical structure may provide an excellent guess, there is a need for a rational approach to the general problem of antibacterial action. This need has been felt for some years (1) and has been filled to some extent by the concept of analogue competition, but unfortunately this is difficult to apply to most of the more potent antibacterial substances. This is perhaps because such a concept does not include the several types of interaction theoretically possible between drug and receptor site, or else that we have not yet discovered the competing metabolite. A more general approach may be the quantitative study of the association between drug and cell, for which the most sensitive and convenient assay is radioisotopic labeling of the drug. This should give information which applies directly to the initial damage. It still appears difficult to introduce enough radioactive atoms into the molecule to detect the small amounts of the more highly active antibiotics bound by bacteria, but fortunately penicillin, unique in many other ways, is an exception in this case also.

The following review discusses the uses and limitations of a rational approach to penicillin mode of action derived from the binding of radioactive penicillin by bacterial cells. It includes an examination of the likelihood that penicillin binding is related to its site of action, together with fresh information and interpretations of older data afforded by the properties of the several penicillin-binding components discovered.

I. THE PENICILLIN-BINDING COMPONENT AS THE INITIAL SITE OF ACTION OF PENICILLIN

Types of Binding Possible and the Binding Found

It seems to be self evident that to affect a living cell some molecules of a drug must be
able to reach, to interact with, and thus to derange a certain cellular system in a manner detectable as a direct association between drug molecules and cell. Such a binding site can be termed the site of action of the drug and may represent the site controlling the reaction first disorganized by the drug. An alternative is that the drug changes the cell's environment in some way. With penicillin such an alternative must involve some quantitative relation between penicillin and a medium constituent, so that any changes in medium should produce much greater differences in penicillin sensitivity in a given organism than have in fact been found (2, 3). Thus this possibility seems most unlikely.

To return to the general case, the binding of drug by the cell may be freely reversible in the sense that bound drug molecules can be rapidly removed by washing or by equilibration with freshly added drug, or the bound molecules may be removed so slowly that the combination appears to be irreversible. The measurement of binding by the site of action may be made difficult by a large amount of both reversible and irreversible binding by other cellular components, which has negligible biological effect. It is also possible that binding by these other sites is necessary to produce the characteristic biological effects, i.e., the site of action is multiple. Even a single site of action may produce many metabolic defects, one originating from another in the form of a chain.

This seemed to be true with penicillin, and to obtain information about the initial stage in the series Cooper and Rowley (4) and Maass and Johnson (5, 6) independently examined the uptake of penicillin by bacteria, using penicillin labeled with S\(^{35}\) by biosynthesis. They confirmed an earlier suspicion (7) that the amount of penicillin bound either reversibly or irreversibly was very small, and Rowley \textit{et al.} (8) demonstrated that little increase in irreversible penicillin binding occurred above very low concentrations. Later use of purer radiopenicillin solutions (11) showed that saturation of these binding sites was practically complete at 0.1 u/ml. Pasinsky and Kastorskaya (9) also found that a similar amount (about 1000 molecules/cell) was lost overnight from a broth supernatant in contact with cells at 37 C, but this corresponds with the amount which can be calculated from the stability curves (10) to be inactivated in a control solution without cells under the same conditions.

The shape of the irreversible uptake curve, of Rowley \textit{et al.} (figure 1) suggested that the cells contained a definite trace component, which, for convenience, has been termed the penicillin-binding component (PBC). Any component which similarly binds penicillin has in this review been termed PBC, although it is recognized that in different organisms it may differ chemically or even have quite different functions. In theory, for any particular organism there may be as many types of binding sites as there are molecules bound (200-750 molecules/cell), but, in general, the sites appeared to be similar in that chemical agents either completely prevented binding or had no effect. This similarity, together with the small amounts bound, suggested that the site of action was not multiple, although the possibility of several irreversible binding sites, only one of which was connected with penicillin action, has been suggested (12, 12a, 6) to account for the lack of correlation between resistance and penicillin bound, in organisms trained to resistance.

\textbf{Purity of the Radiopenicillin Preparations}

That “PBC” was binding penicillin and not radioactive impurities was regarded as proved.
(11) by: (a) an identical uptake curve with a radiopenicillin preparation containing less than 5 per cent of its $^{35}$S in impurities, so that the $^{35}$S bound was more than the total amount of impurity present; (b) rapid prevention of all uptake by pretreating the cells with 0.1 u/ml ordinary crystalline penicillin; (c) destruction of the horizontal portion at low concentrations by inactivating the drug beforehand with the specific enzyme penicillinase (figure 1). Pollock and Perret (13) distinguish between a "specific" penicillin binding, similar to that obtained using purified radiopenicillin and a "nonspecific" binding, which may be due to nonpenicillin $^{35}$S impurities. However, other workers (5, 6, 12, 12a, 14, 15, 16) do not appear to consider the effect of small amounts of nonpenicillin $^{35}$S impurities. These can be strongly bound by cells and be present in considerable amounts even in "crystalline" radiopenicillin preparations (11), giving uptake curves with a marked upward slope on sensitive organisms (figure 1). This upward slope is sufficient to mask "specific" binding of $^{35}$S completely at higher concentrations, and can be due to penicilloic or penillic acids, and possibly others of the many solvent-insoluble penicillin degradation products. It is essential to be sure that "penicillin" binding is in fact due to penicillin when considering the biological effects of the drug, and a good test of this appears to be the horizontal nature of the uptake curve with certain organisms, or the lack of effect on the uptake curve of absorbing the penicillin preparation with a small amount of the test organism before measuring its uptake.

Correlations Between Penicillin Binding and Its Antibacterial Action

From the point of view of the possible initial site of action the irreversible PBC proved interesting because of its several properties which fitted in well with known aspects of penicillin action. These were:

a. PBC occurred in penicillin-sensitive strains, but resistant strains previously selected as free from penicillinase were either without PBC or sheltered it from penicillin at low penicillin concentrations. There was a correlation between penicillin bound and sensitivity (8). This was not the experience of Maass and Johnson (5), but Eagle (12, 15) confirmed and extended the work of both Johnson and Rowley and their colleagues, showing that naturally occurring penicillin-resistant strains were able to bind penicillin to an extent varying with their sensitivity, although artificially trained strains were not. Thus, with natural resistors the final uptake was always of the same order when the penicillin was at equi-effective (LD_{50}) titers.

b. At low extracellular concentrations sensitive cells bound penicillin so that the intracellular concentration was greater than the extracellular (8). This concentration of penicillin by the sensitive cell occurred only over the minimal inhibitory range (0.01-0.1 u/ml) (8) and, because of the low collision frequency, the rate of penicillin binding was sufficiently slow in this range to be of the same order as the rate of bacterial growth (6). This prompted the interesting hypothesis that the minimal inhibitory titer was the result of a balance between rate of binding at this concentration and rate of PBC resynthesis, rather than of a removal of penicillin from the cell by dissociation of the PBC-penicillin complex at low concentrations.

It will be mentioned later (p. 37) that PBC appears to be made available to penicillin by two co-existent mechanisms: (i) normal synthesis = rate bacterial growth in mg/ml/min X PBC content in u/g; (ii) "turnover" = total rate of exposure less the total rate of synthesis. It will be seen that the rate of binding has only to exceed slightly the rate of synthesis, rather than total exposure, for the cell to be doomed by slow annihilation of its free PBC. Under certain conditions (18) normal synthesis was 0.2 u/g/30 min. It is therefore perhaps further evidence for the hypothesis of Maass and Johnson that the rate of binding at the minimum inhibitory concentration (0.02 u/ml) by the same organism under slightly different conditions (19) was observed to be 0.6 u/g/30 min, which is of a similar order to the rate of PBC synthesis actually observed.

c. Pretreatment with crystalline penicillin prevented all uptake but cetyltrimethylammonium bromide, Aerosol "OT" and Tween 80 had no effect (8, 20). These detergents, together with penicillin, failed to remove bound penicillin. Thus binding by PBC was not just a nonspecific surface adsorption, but was specific to the penicillin molecule, an essential observation if PBC is to be the site of action of penicillin.

d. The penicillin bound increased on growth in the presence of penicillin (6, 8, 18, 21), and it is known that penicillin is lethal only under
conditions of growth. The implications of this finding are discussed in section V. In disagreement with these observations, any increase of binding on growth was regarded as within experimental error by Eagle (15).

e. At 0.1 u/ml the rate of binding of penicillin by PBC became almost instantaneous, so that PBC was rapidly saturated above 0.1 u/ml but required a measurable time to reach saturation below this level. The rate of killing by penicillin also increased up to 0.1 u/ml (17) but stayed constant above this titer until the rate began to drop at high concentrations. This optimal concentration (0.1 u/ml) is again the lowest at which production of fresh viable units is stopped immediately by adding penicillin (18, 22).

f. The uptake by PBC is sufficiently small to allow the well-known lack of effect of inoculum size on penicillin activity.

There is thus a considerable amount of evidence which strongly suggests that PBC, the irreversible penicillin-binding component, is in fact the initial site of action of penicillin, although it seems a particularly difficult connection to prove in spite of the fact that it seems to be the only association of penicillin with the cell having the necessary properties. Little work has been done on possible reversible binding of penicillin, and indeed evidence that it exists is rather indirect, as shown in the next section.

II. PERMEABILITY OF THE BACTERIAL CELL TO PENICILLIN

If it could be shown that penicillin cannot penetrate sensitive cells, then the search for the initial reaction disorganized by penicillin could be confined to the part of the cell external to the osmotic barrier. On the other hand, if it can be shown that penicillin can penetrate sensitive cells, then impermeability becomes a possible mechanism of resistance. For these reasons it is proposed to discuss this question a little more fully than would otherwise be necessary.

Experiments involving the addition of penicillin in known amounts to thick cell suspensions (5) suggest that the cell is freely permeable to penicillin, and these results have been confirmed in our laboratory using a Cowan's type II strain of staphylococcus. However, this type of experiment involves the assumption that even at very high concentrations negligible amounts of penicillin are reversibly bound by the cell. The irreversible binding (by PBC) can be allowed for.

The fact that the sonic cell-extracts sedimenting between 27,000 and 144,000 X G bind penicillin to the same extent as intact cells (15) does not necessarily mean that the cell is permeable to penicillin. In fact, as this fraction would not include the particulate fraction which contains 75% of the PBC (23), and should therefore bind less penicillin than intact cells, this is evidence similar to section b below that the cell is impermeable to penicillin. As PBC appears to reside at or near the surface of the cell (23), penicillin need not be able to penetrate the intact cell completely in order to reach PBC, and in fact complete permeability of the cell with little reversible binding is unexpected in view of the following experimental findings:

a. Similar cells are not freely permeable to smaller negatively charged ions such as glutamate, aspartate and phosphate (24, 25, 26).

b. The unfraccionated cytoplasmic contents liberated on rupture reversibly bind very much larger amounts of penicillin than do intact cells (23). This high binding is to be expected from the proteins present (27), although it was not found in the fraction sedimenting between 27,000 and 144,000 X G (15). Although penicillin is not surface active (28) at least some reversible association with the proteins of the cell surface as well as the interior might be anticipated from the work of Klotz et al. (27). If the intact cell were freely permeable to penicillin, this would lead to the expectation that reversible binding should be much higher than was actually observed, although it is possible that many binding sites are only exposed when the cellular proteins are disarranged by rupture.

c. The cell contains some reserve PBC which is not made available to penicillin until after rupture in a fashion which does not suggest enzymic synthesis (23). Thus some mechanism to protect this reserve PBC from penicillin must be postulated.

d. The qualitatively different effect of penicillin at high concentrations (17) implies either greatly increased binding at high titers, or a greatly increased rate of penetration of penicillin in order to reach sites not previously available. Neither of these concepts can be reconciled with the idea of little reversible or irreversible binding (even at high concentration) accompanied by rapid and complete permeability (even at low concentration).
On the other hand, it is rather a coincidence that the amount presumed to be reversibly bound is just enough to cancel out the increase in concentration to be expected from the exclusion of penicillin from an impermeable cell in the thick-suspension experiments mentioned. If the volume impenetrable by penicillin is the same as that impenetrable by phosphate [about 5 ml/g dry wt for the Staphylococcus aureus (Micrococcus pyogenes var. aureus)] used by Cooper (18), then the reversible binding of penicillin by this organism cannot be more than 0.5 u/g at 0.1 u/ml and is directly proportional to concentration. This, like the irreversible binding, would be small enough to render incoculum size of no effect on penicillin activity. Also, as pointed out by Maass and Johnson (5), some evidence of saturation might be expected at 10,000 u/ml, although it is not necessarily true to say that the amount of penicillin which can be adsorbed outside the osmotic barrier must be limited to a single monomolecular layer, as the part of the cell outside the osmotic barrier may have considerable depth (26, 29) with many potential reversible penicillin binding sites. It is interesting that this depth [about 20 m for S. aureus, 26] is the same as the thickness of the cell wall liberated on mechanical rupture (30), and may be identifiable with the space occupied by the cell wall (29).

The question of permeability is therefore still open, but it would seem likely that the intact cell does bind a little penicillin reversibly but is not readily permeable to penicillin. If so, PBC either resides at the external interface of the osmotic barrier or within that small metabolic zone which may exist outside the osmotic barrier but within the confines of the cell wall (29).

One must ask if such reversible binding, rather than PBC, causes the lethal effect. This is difficult to investigate experimentally but, as has been already seen in Section 1, many properties of PBC fit in well with the observed effects of penicillin. The reversible binding, if it exists, could show no marked change at a particular concentration such as the minimal inhibitory titer. Other obvious relations between reversible binding and lethal action of penicillin have not been sought. It is interesting that Saccharomyces cerevisiae cells were impermeable to penicillin and did not bind the drug reversibly or irreversibly (5), and Bacillus cereus spores appear to be impermeable to penicillin in that PBC is somehow not available for reaction with penicillin (31). With yeast, at least, the question of impermeability was not obscured by any significant reversible binding. Considerable amounts of S²⁰ bound by Escherichia coli K12 from high concentrations of radiopenicillin could be removed by washing, so that penicillin was assumed to penetrate K12 and to be inactivated intracellularly by that organism (15), and it seemed likely that mammalian cells were permeable to penicillin (32). Nevertheless, these data do not permit a strict decision as to whether penetration or reversible adsorption accounts for the penicillin which can be eluted.

III. THE CHEMICAL PROPERTIES OF THE PENICILLIN-BINDING COMPONENT

The most likely interpretation of the observations that certain bacteria apparently bind penicillin specifically is that these cells contain a limited amount of a substance, 'PBC', which should be definable in chemical terms. It has been already noted that the experimental evidence is in favor of this being the site of action of penicillin. Therefore, it should greatly help us to understand the mode of action of penicillin if we knew the chemical nature of PBC; an essential stage in its purification must be its isolation from the cell in a soluble form. There are likely to be difficulties in its purification, as it may be a large molecule rather than an easily removable cofactor, thus involving removal of a very high proportion of chemically similar impurities, or it may be the result of the conjunction of two large molecules between which the penicillin happens to fit. Any attempt to separate the two large molecules would then naturally lead to the disappearance of PBC. Nevertheless, several cell-free preparations have been obtained by mechanical cell rupture which can bind penicillin in a similar manner to intact cells, and can thus be presumed to contain PBC (15, 16, 21, 23, 31).

Little success has been obtained in characterizing this component, and nothing is known with certainty of its chemical nature. Daniel and Johnson (16) find that the 'specific' binding is not destroyed by trypsin and Cooper (19) finds that it is not destroyed by trypsin, lecithinase, or ribonuclease. Cooper (23) has shown that, with the particular staphylococcus he used, the PBC is concentrated in a small particulate fraction.
which contains about 10 per cent phospholipid and 10 per cent other lipid, the remainder (protein plus a polyglycerophosphate complex or PGP) being material very similar to the cell wall in composition (39). This PBC-lipid-protein-PGP complex was not fractionated by several mild techniques, and both the lipid and PBC appear to be strongly bound, producing a close correlation between lipid P, PBC and penicillin labeled with C14 which had been attached before rupture. Solvent treatments which had no lipid-phosphate extracting effect did not remove PBC, but PBC was not found in any fraction after extraction procedures which were sufficiently drastic to remove phospholipid—so that PBC appeared to be destroyed by these procedures. However, cells grown in radiopenicillin, washed, and treated with 90 per cent phenol lost their lipid but not their S35, suggesting that PBC may be more closely associated with the protein-PGP moiety than is the lipid. Eagle (15) found penicillin binding by the large-molecular material sedimenting at 144,000 × G but binding was to the same extent as intact cells, showing no concentration of PBC in terms of dry weight. This material had previously been centrifuged at 27,000 × G, when the cell walls and the PBC-lipid-containing particles would have been removed, leaving only the soluble portion of the lipid particles with a large amount of soluble protein. Considerable 'nonspecific' binding was suggested by the rate of increase of uptake with concentration.

PBC appeared to be rather unstable in the intact cell or in the cell-free state, being rapidly destroyed by acid and moderate heat (16, 19). Even at the optimum pH and 2 C, 25 to 50 per cent was lost overnight. It can be seen that this character of instability, when coupled with the very low solubility of the only soluble preparation obtained (the lipid-containing particles), renders further chemical purification difficult, and a more efficient and stable solvent system would seem to be a prime requirement.

IV. THE NATURE OF THE REACTION BETWEEN THE PENICILLIN MOLECULE AND PBC

Knowledge of the means, whether chemical or physical, by which PBC is bound by penicillin is important for the design of other antibacterial agents that may function similarly to penicillin and yet may penetrate the cell more easily or be unharmed by penicillinase. Also such knowledge should contribute information on the constitution of the penicillin binding component and on the penicillin mode of action.

The curve relating penicillin uptake by sensitive bacteria to concentration in the suspending fluid (figure 1) is that to be expected from a rapid reaction irreversibly saturating a component present in small amount. The binding of penicillin has been mentioned already to be very firm indeed, as penicillin S35 cannot be removed, once attached, by fresh penicillin, copious washing or treatment with anionic, cationic or neutral detergents (5, 8, 15, 20). Pretreatment with detergents does not affect binding (except in the special case of phenol), but binding is completely prevented by small amounts of crystalline penicillin added beforehand. Thus, as penicillin is also only feebly surface active (28), binding does not appear to be by adsorption at a surface in similar fashion to detergents. An alternative mechanism would be by van der Waals' attractive forces similar to those presumed to bind antibody to antigen or haptene, but most antigen-antibody combinations have an appreciable degree of reversibility.

It is perhaps more likely with a reactive molecule such as penicillin that the PBC-penicillin complex is formed by chemical reaction. If so, unlike a physical type of binding, the penicillin once fixed has changed chemically and is no longer penicillin. The correlation between amounts bound of S35 and C14 labeled in the position indicated below shows, however, that the molecule, even if altered, is not split between the S and C14 atoms (15).
A clue to the nature of the binding reaction may be gained from a consideration of the parts of the penicillin molecule which can be altered or dispensed with without gross loss of activity. This shows that: (a) The nature of the R radical is in general unimportant, as many different substituents can be used without loss of activity. The α-amino residue in cephalosporin N is an exception to this (33). (b) The carboxyl group and thus the negative charge on the molecule are not vital, as penicillin amide (34) is at least 15 per cent as active as penicillin against sensitive species. This has been confirmed in our laboratory. As the activity is more resistant to penicillinase than penicillin itself, the active form of the amide is not free penicillin, as may be the case with the diethylaminoethyl ester (35). (c) Any penicillin derivative not possessing both the β-lactam or thiazolidine rings intact are biologically inactive. This supports the findings (16) that several penicillin derivatives (peniciloic, penilloic, penillic, penillenic and penillionic acids, desthiopenicillin, cysteinepenicillin and penicillamine which all lack one or other of these structures) do not prevent penicillin uptake by themselves binding PBC. Thus the important part of the molecule appears to the central part, and it is probably significant that practically all the reactions rapidly undergone by penicillin at 37 C and neutral pH are acylations performed by the splitting of the β-lactam bond, e.g.:

\[
\begin{align*}
\text{R-CO-NH-CH—CH—COONa} & \quad \text{or} \quad \text{HO—H} \\
\text{R—CO-NH-CH—CH—COO} & \quad \text{or} \quad \text{H2N—COOH}
\end{align*}
\]

As the thiazolidine ring appears to be rather unreactive chemically, the implication of these findings is that penicillin acylates its receptor PBC by a reaction which splits the β-lactam ring. This is supported by the finding that pre-treatment of the cell in strongly buffered suspension with a small amount of acetic anhydride or acetyl chloride, but not acetic acid, completely prevented penicillin uptake (8). Also a mild alkaline treatment of the radiopenicillin-cell complex extracted the S\(^{35}\) as penicilloic acid (35a), a result to be expected if penicillin is bound intact or by acylation. Penicilloic acid itself is only slightly bound (11), so that one would not expect the bound S\(^{35}\) to be in this state before alkali treatment.

It may be argued that if the β-lactam ring is the functional group, then the rest of the molecule should not be necessary, but a reactive molecule such as penicillin would probably need some means of specifically limiting its reactivity, such as by steric hindrance, so that at high dilution the penicillin is not wasted by acylation of sites that are not essential for the cell. Another function of the thiazolidine ring is likely to be to activate the β-lactam system, as other substances containing the β-lactam are much less reactive chemically as well as biologically. It is possible too that PBC is a substance which is extremely easy to acylate, perhaps undergoing reversible acylation in the bacterium, and penicillin represents a compound of intermediate acylating ability, a compound that is without the wasteful reactivity of acetyl chloride, but is more reactive than the β-lactam ring alone. A search for a similarly intermediate type of acylating agent that is simpler chemically than penicillin would represent a new approach to a synthetic penicillin and may reveal a substance which is cheaper, less susceptible to penicillinase or more capable of penetrating the resistant bacterial cell but still able to bind PBC fairly specifically.

If penicillin is in fact bound by acylation, then the likely receptor groups are —OH, —SH or —NH\(_2\). The facts that penicillin reacts preferentially with the —NH\(_2\) group of cysteine rather than the —SH (36) and that pretreatment of the cell with formalin has no effect on penicillin binding (21) do not necessarily rule out —SH or —NH\(_2\) as receptors. Daniel and Johnson (16) have reported the interesting finding that their PBC may have to be in an oxidized state to bind penicillin. Perhaps the 50 per cent of the total PBC which is not available to penicillin in intact resting cells (23) is in the reduced form rather than sheltered within the cell, and the increase of bound penicillin during growth (18) is due to the progressive oxidation of this group in the presence of penicillin as part of a routine oxidation-reduction cycle. Thus the increase in available PBC in cell-free preparations on standing (19) may be due to its oxidation in air.
The finding that penicillin is more active at pH values slightly lower than 7 has led to the suggestion (37) that penicillin competed with the hydroxyl ion for its site of action. That this is not the case is shown by the irreversible nature of the penicillin binding, the activity of penicillin amide (34), and the lack of effect of pH on the amount of penicillin finally bound (19), although the rate at which penicillin was bound was sufficiently increased at a lower pH to account for a proportion of the increase in activity.

The rather small effect of pH on rate of penicillin uptake suggests that penicillin may react with the cell in the ionized (lipid insoluble) form, an idea which is not supported, however, by the findings that the penicillins, [e.g., most azopenicillins (38), the natural penicillins and cephalosporin N (33)] are active roughly in the same order as their lipid solubility. Perhaps lipid solubility assists penetration into the cell.

It is interesting to calculate in this respect that, at pH 6.7 and 0.02 u/ml, the volume occupied by each cell would normally contain 25 penicillin molecules, but only 1 in 25,000 of these would be un-ionized. The reacting molecules have to find positions at or near the cell's surface, but covering only 10⁻⁶ of that surface. It is evident that, even for the ionized molecule, the chances of effective contact are small. At pH 6.7 and 0.02 u/ml the penicillin binding rate is of the order of 100 molecules/cell/hr (19).

V. THE NATURE OF THE PROCESS FIRST DAMAGED BY PENCILLIN IN SENSITIVE BACTERIA

If PBC is the site of action of penicillin, then it is probable from the small amounts in bacteria (estimations of the molecules of penicillin bound/cell vary from 80 to 1600) (5, 13, 15, 18), that PBC is part of some catalyst, whether enzymic or not, whose activity is changed or halted by the binding of penicillin. The amounts of penicillin bound are similar to the amounts in bacteria of vitamins (40) such as pyridoxin (2100 to 6600 molecules/cell) and folic acid (180 to 1200/cell).

A possible alternative is that the PBC-penicillin complex is itself the toxic substance and blocks some other process, but this is not considered here, as too little is known of this complex to help us study a second unknown. For the purpose of discussion, then, it is assumed that PBC plays an active and vital part in the cell's economy, and damage to the reaction governed by PBC is the initial effect of penicillin. This may be because the cell requires the product of this reaction, or because the precursor to the product accumulates and is toxic (41).

Location in the Osmotic Barrier as a Possibility

A clue to the location in the cell of the penicillin site of action may be the presence of PBC in the particulate lipo-protein fraction liberated on cell rupture (23). Of the soluble material so liberated in distilled water, only this fraction with little other dry weight was retained in the cell wall fraction when formalin was added to the medium before rupture began. This observation suggests its proximity to the cell wall in the intact cell. Mitchell and Moyle (39) believed from chemical assays that it may represent the lipid layer observed cytologically under the cell wall. Also, penicillin uptake was prevented in a manner which was closely parallel with osmotic barrier destruction by pretreating the cell or the lipid particle fraction with dilute solutions of the neutral lipid-soluble detergent, phenol, whereas lipid insoluble detergents had no effect on penicillin uptake (20).

This association between PBC and cell wall lipo-protein suggested that penicillin may initially involve some function of the osmotic barrier, generally believed to have lipoidal properties and necessarily lying close to the cell surface. As the osmotic barrier controls many functions, some generalized damage to it could account for the many changes induced by penicillin.

It is curious that, if one excepts cells that are resistant because of penicillinase production and the penicillin-impermeable yeasts (see p. 32), there is nearly always a close correlation between ability to retain the basic dye in the gram staining procedure and penicillin sensitivity. There is also a correlation between gram positivity and (a) content of a polyglycerophosphate ester (45a, 49) which is bound partly to cell wall protein and partly to the underlying lipo-protein membrane (39) and (b) lipid P content. This correlation led Mitchell and Moyle (45a) to suggest that a cell-wall lipid-polyglycerophosphate-protein component (lipid particle fraction?) may be a common structural feature of all gram positive bacteria. There is also a difference in the osmotic character of gram positive and gram negative cells which presents itself as a greater rate of loss.
of small molecular components in distilled water suspensions of the gram negatives (45a), but there is no correlation with nucleic acid content. It is possible but not yet well demonstrated that the difference in osmotic character may also include active transport mechanisms for certain solutes (26, 44).

These observations have suggested (45a) two co-existing mechanisms for gram positivity: (a) an ionic mechanism predominating in gram staining without a mordant, which would explain the differences in pH at which the basic dye is eluted from positives and negatives ("iso-electric points”), (b) a mechanism superimposed by use of a mordant which appears to retain more dye than the ionic mechanisms, so that differentiation is sharper, and which is somehow involved with the cell wall. This mechanism may be a greater retention by the cell wall of gram positives of the poorly soluble dye-iodine complex, as ruptured cell preparations retain very little dye. In considering the coincidental correlations mentioned, the reviewer finds it difficult to escape the belief that the staining difference noticed by Gram happens to reflect a fundamental difference in cellular physiology related to the cell wall which includes the normal function of PBC. Some effects of penicillin on cell wall activities are discussed in reference 23, as well as elsewhere in this review.

However, although this concept provided some guide for further research, the multi-functional nature of the structure called 'the osmotic barrier' itself means that there are many possible characters which could be inactivated by penicillin. Such possibilities include:

a. Synthesis of its own material, perhaps including lipid phosphates, and of the overlaying cell wall. Its intact condition is necessary to retain those solutes already present in the cell.

b. Transport into the cell of metabolites which cannot penetrate freely.

c. Metabolism at the outer surface of the cell of substances which do not penetrate.

d. Since the osmotic barrier must also have an inner surface, this structure may have metabolic activities inside the cell which may not be recognized as functions of an osmotic barrier. It would therefore be interesting to know whether or not the cell is permeable to penicillin (see section II). An argument against this is that as penicillinase is secreted rather than liberated by cell lysis (42), the final stages of penicillinase synthesis should proceed at the outer rather than the inner surface of the osmotic barrier, as otherwise one has the paradox of an osmotic barrier which is permeable to proteins but not to amino acids. Thus the close connection of PBC with penicillinase synthesis in Bacillus cereus (see section VIIa) suggests that PBC also functions at the external surface. On the other hand, Pollock (31) suggested that penicillinase synthesis was induced inside the cell as PBC was included in spores formed within the cell wall. However, PBC appears to reside in the lipid material near to but easily detached from the cell wall (23) and this probably is included in the spore. Thus PBC could be beneath the structural cell wall yet at the same time at the external osmotic surface of the cell, as this is deep to the cell wall surface (26).

Consideration of Early Effects of Penicillin

Cooper (18) examined the effect of penicillin on certain osmotic barrier functions. His results suggest that there may be in fact a serious disturbance of active osmotic properties, as at least one and perhaps all of the transport mechanisms had abruptly ceased to function before many other of the principal activities of the cell, such as glucose oxidation and fermentation, protein or peptide and nucleic acid synthesis. The cessation of glutamate assimilation (43) at a similar time would also lend support to this idea, but it is not at present as likely as it had seemed earlier that glutamic acid assimilation is an active function of the osmotic barrier (44). The accumulation of acid-soluble nucleotides noticed by earlier workers (45, 46, 47) was suggested (18) to be due to a secondary acid-lability in the RNA, perhaps caused by osmotic barrier malfunction, rather than to a direct interference with RNA synthesis, as extraction with dilute phenol in place of trichloroacetic acid revealed no accumulation of nucleotides. Similar nucleotides accumulate in resting cultures and cultures treated with other antibiotics (47a) which also suggests that this may be simply a manifestation of unbalanced growth.

Unfortunately, none of these disturbances occurred sufficiently soon to be incriminated as the site of action of penicillin. The literature reports many metabolic defects which have been suggested, with varying degrees of caution, as the site of action of penicillin. However, since
the ultimate death of the cell will stop very many reactions, it is important to place the defects in some sequence, in which, by definition, the site of action is the first. Not many early defects of penicillin have been noticed, but of these the accumulation of ribonucleotides (45), drop in surface charge (48), and drop in cell-wall polyglycerophosphate (49) all appeared to start at once. If these are not all related, then in this organism and under these conditions the sequence of defects is very rapid indeed. The different strains and conditions used by Cooper (18) permitted some separation of metabolic disorders induced within an hour by penicillin, particularly uptake of P and metals, but none of these occurred at once. The experiments used penicillin at 0.1 u/ml, the lowest concentration in which saturation of PBC is complete within 2 min, the bactericidal action is most rapid (17), and production of fresh viable units is stopped immediately (22), so that the initial lesion should be evident practically at once.

Thus, although, (a) a correlation exists between penicillin sensitivity and some cell wall properties related to gram positivity, (b) PBC resides in or close to the osmotic barrier, and (c) many of the earliest changes appear connected with active cell wall functions, yet there is a time lag before the earliest change appears, so that the location of the initial lesion is still not known. The intact nature of the osmotic barrier was not affected until considerably after these changes, nor was synthesis of lipid phosphate or the 'lipid particle' fraction.

Increase of Penicillin Uptake when Growth Occurs

Rowley and his colleagues (8, 18, 21) and Maass and Johnson (6), but not Eagle (15), found that the penicillin bound progressively increased when growth occurred in the presence of penicillin to a value about double that of resting cells. This was shown (18) to be due to the exposure of a reserve of PBC equal in amount to the 'free' PBC which was not normally available to penicillin (23), rather than to an increased rate of synthesis of PBC. It could not be due to an increased rate of reaction between penicillin and PBC per se, as this was already very fast (complete in 2 to 3 min) compared with rate of binding observed (complete in 30 min). Thus the rate of PBC exposure was the rate-limiting step. It was suggested that this exposure of reserve PBC occurred as part of a routine cycle while PBC was metabolizing its normal substrate S (figure 2), and as soon as PBC was released from its duties with S it was immediately bound by the penicillin present. Thus the rate of penicillin binding above that due to total synthesis of PBC ("reserve" + "free") may be a measure of the rate of turnover of PBC. It was calculated on this basis that PBC metabolizes 3000 molecules of S for every cell produced, or about 10 molecules per hour per molecule of penicillin bound. Such a turnover rate is rather small compared with many enzymes but is of similar order to certain trace reactions such as synthesis of pantothenate in Pseudomonas aeruginosa (40). The figure of 3000 molecules/cell could probably be up to 10 times larger, but even so a very specific analytical method would be needed to detect the quantitative change brought about by addition of penicillin. Thus it should be of little avail, for example, to look for a change in over-all rate of nucleic acid synthesis.
Reactions that Are Not the Function of PBC

The very small "turnover rate" of PBC, the effectiveness of penicillin in the absence of air or all organic matter other than glucose, and the likelihood that, above 0.1 u/ml, the use of PBC is completely stopped very soon after the penicillin is added, were suggested (18) as indications of what are probably not the functions of PBC, namely: (a) gross assimilation of O₂, Ca^{++}, Na^{+}, Mg^{++}, K^{+}, PO_{4}^{3-}, Fe^{+++}, or Co^{++}, organic substances or other substances contributing to the dry weight; and (b) synthesis of the major part of lipid phosphates and the lipid particle fraction, and cell wall polyglycerophosphate, proteins, nucleic acids or intermediates. However, PBC may assimilate or synthesize a small fraction of these substances, or assimilate a trace substance required in amounts similar to that of cobalt (about 10⁴ atoms/cell). Alternatively, the suggestion that PBC may be capable of reversible oxidation-reduction mentioned in section IV may mean that PBC is concerned with the energy source of a very small reaction, but this is presumably not at the stage of oxygen utilization. It may be that the suggested simultaneous failure of a number of active functions of the osmotic barrier is due to the initial breakdown of some common factor such as an energy supply localized in the osmotic barrier. Further progress is hindered by the following obstacles: (a) isolation and chemical definition of PBC and demonstration of some catalytic activity to the exclusion of others, and (b) demonstration of a very small catalytic activity, requiring a very sensitive and selective assay method, which probably occurs at the cell surface, and which is completely and very rapidly stopped by very low concentration of penicillin.

VI. HYPOTHETICAL TIME TABLE OF METABOLIC EVENTS FOLLOWING ADDITION OF PENICILLIN

Penicillin causes many defects in the cell's metabolism, any one of which—for example, blockage in phosphate or glutamate uptake, shortage of Mg^{++}, Na^{+} or K^{+}, damage to RNA and protein synthesis, inactivation of free PBC or inability to synthesize more PBC—is sufficiently serious to merit the title "cause of death." Thus it is of little value to nominate as the cause of death any one stage of the chain of defects induced by penicillin except perhaps the first, and it is more desirable to attempt the considerable task of enumerating the defects in the order of their occurrence. This is clearly impossible to do in detail, but as has been already pointed out, some such scheme is necessary to describe fully the "mode of action" of any antibiotic.

The various properties of the penicillin-binding component which have been outlined above, together with observations in the literature on the effects of penicillin on bacterial growth, suggest a sequence for the events which are induced by penicillin in those fully sensitive cells inhibited by less than 0.1 u/ml, and it may be helpful to summarize this inferred sequence as a timetable starting with addition of penicillin to a growing culture. Inferences followed by a query have little or no direct supporting evidence.

<table>
<thead>
<tr>
<th>Inferred Event</th>
<th>Supporting Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time: 0-2 minutes</strong></td>
<td></td>
</tr>
<tr>
<td>a. Above 0.1 u/ml and below those concentrations where secondary effects may be expected, penicillin reacts with and firmly fixes all free PBC at once (displacing its normal substrate S?). Metabolism of S ceases at once when all free PBC is fixed?</td>
<td>a. Examination of rate of penicillin binding at different penicillin concentrations.</td>
</tr>
<tr>
<td>b. At the minimal inhibitory level (e.g., 0.02 u/ml) the lower rate of inactivation of PBC is just equal to its rate of resynthesis. When the rate of inactivation of PBC exceeds the rate of synthesis, the rate of utilization of S declines?</td>
<td>b. Firmness of binding penicillin (5, 6, 8) eliminates dissociation of PBC-penicillin complex as cause of minimal inhibitory level. Rates of binding and of growth are comparable at minimal inhibitory level; calculated equivalence of penicillin binding at 0.02 u/ml and PBC synthesis (see paragraph b on p 30).</td>
</tr>
<tr>
<td>c. Production of fresh viable units as measured by</td>
<td>c. Equivalence of lowest concentration (0.1 u/ml)</td>
</tr>
</tbody>
</table>
viable counts ceases when all free PBC fixed (i.e., when metabolism of S also ceases?).

d. Other metabolism is unaffected for the time being.

Supporting Evidence
where all PBC is fixed within 2 min (6, 19) and lowest concentration where viable unit production is stopped at once (18, 22).
d. Numerous references discussed in section V.

**Time: 2–30 minutes**

a. This is a period of bacteriostasis.

b. Above 0.1 u/ml, additional PBC is exposed and is fixed at once by penicillin (on being released from its duties with S?).

c. If penicillin is removed before the last of the reserve PBC is freed (i.e. before 30 min) (18), then this residual PBC enables growth to begin again at once.

d. Less than the optimum amount of PBC is then immediately available to the cell.

**Time: 30–60 minutes**

a. Viable counts begin to decrease in exponential curve if penicillin still present at this time (50).

b. Irreversible damage to the cells is completely prevented if growth is stopped within 5 min by helvolic acid, but after 30 min no protection is afforded (51).

c. Penicillin bound per unit volume of culture still increases, but the rate steadily diminishes (18).

d. The cell ceases to accumulate gross dry weight, Na+, Mg++, K+, phosphate (18) and glutamate (43) during this period, although other metabolism has continued.

e. After 30-min contact, there is a lag before growth commences which is longer the longer the time spent in contact with penicillin (22).

f. Removal of penicillin at any stage prevents further cells dying (22).

g. Uptake of Na+, Mg++, K+, phosphate and dry weight (18) and glutamate (43) cease, cell wall material (49) and cell protein (52) are no longer synthesized, although nucleic acid and peptide syntheses continue. Total cobalt and iron uptake and total lipid P synthesis are unaffected (18).
Inferred Event

**Time: 60-75 minutes**

Nucleic acid and peptide syntheses slow. Many reactions can be expected to be affected by shortage of metals, phosphate and glutamate and perhaps other nutrients.

**Time: 75 minutes**

The osmotic barrier begins to lose its osmotic properties and the dissolution of the cell commences.

On this hypothesis there appear to be two reasons for penicillin requiring active growth to be bactericidal: (a) the reserve PBC has to be removed by continued exposure to penicillin during growth, and (b) the cell must also lose its ability to resynthesize the PBC by continued metabolic activity in a deficiency of many substances either by exhaustion of an essential component (e.g., metals or phosphate) or by accumulation of a toxic product. When this happens, the cell cannot again obtain PBC, and since by definition PBC is essential, it can from this moment be regarded as nonviable. Growing in a deficient medium without penicillin would not be expected to be bactericidal, as the reserve PBC would not be affected, and would be present to rescue the cell when transferred to a full medium.

It will be recognized that this approximate time-table is very incomplete and contains many presumptions, and that the experimental findings do not necessarily exclude alternative hypotheses. Nevertheless this sequence is suggested as one of the simplest explanations of most of the known data. Some qualifications are necessary, however, in that most experiments have to be performed on very large populations. It is not known, for example, whether 50 per cent of the resting cells take up 1.8 u/g of penicillin and 50 per cent none (so that the doubling of uptake on growth represents exposure of PBC in half the cells only), or whether all take up 0.9 u/g, although this may not matter for the present argument. It is possible also that the sequence of events differs somewhat in different organisms and events may follow each other more rapidly in some, as the onset of swelling occurred at once with one organism (45) but not for an hour with another (18). It seems likely, though, that the initial site of action is very similar in all sensitive species provided that the penicillin is only acting at minimal levels. It has been shown that many other defects commence at levels 100 to 1000 times the minimal concentration (see section VII).

### VII. THE REASONS FOR PENICILLIN RESISTANCE

Demerec (53) has shown that the training of organisms to penicillin resistance results in a series of small increases of resistance rather than one large one, and when sensitive pneumococci are transformed by deoxyribonucleic acid from resistant pneumococci a degree of resistance is acquired which is intermediate between that of the sensitive and resistant organisms employed (54). Strains which have changed their penicillin resistance by a mechanism which is very likely to be but a one-step mutation have nevertheless changed simultaneously in other characters, particularly growth requirements (55, 56).

These findings can be interpreted most simply as indicating that penicillin resistance is controlled by several genic factors. If penicillin sensitivity is caused by possession of a vital component, PBC, which the penicillin molecule must reach and react with, it can be shown that an organism may be resistant to penicillin by a mechanism which is very likely to be but a one-step mutation have nevertheless changed simultaneously in other characters. Some of these ways may each vary quantitatively giving many possible degrees of penicillin resistance, and even individual cells may differ in their penicillin sensitivity (57). It is therefore clear that the mechanism of resistance of any particular strain may be complex, and should be considered individually on its merits.

The information provided by a study of the penicillin binding component on the different causes of penicillin resistance is discussed in the following section.

### Possession of the Means to Destroy Penicillin

This means is thought of as a type of enzyme collectively termed penicillinase, which in one extracellular state has been shown to hydrolyse...
penicillin to penicilloic acid (58). It occurs both extracellularly and intracellularly in many types of organisms, and it is possible that the various forms are not chemically identical and form different degradation products (59). As it is easy to see how an otherwise sensitive organism is nevertheless not killed if the drug can be eliminated faster than its site of action is inactivated, it is not proposed to discuss this mechanism further.

There is, however, an extremely interesting connection observed by Pollock between the penicillin-binding component and the means by which the cell can increase its ability to destroy penicillin. This connection is important with regard to penicillin's mode of action as well as to the more general topic of protein synthesis. Penicillinase synthesis can be strongly stimulated in Bacillus cereus by contacting the cell with penicillin at 0 °C and removing the excess by thorough washing before incubation (42). This stimulation is likely to be caused by some penicillin product which is still attached to the cell when incubation begins, as there has been little chance for irreversible damage such as disturbance of RNA synthesis [which should affect protein synthesis (60)] during the period at 0 °C. It is possible that some penicillin molecules which happen to be reversibly bound cause some damage which remains after the reversibly bound penicillin has been washed away, but stimulation of penicillinase synthesis was marked at 0.0008 u/ml, at which concentration reversibly bound penicillin should be very small (if Staphylococcus aureus and Bacillus cereus are comparable, reversible binding should be less than two molecules/cell).

The stimulation appears to be due to the penicillin irreversibly bound as there is a close correlation between the rate of penicillinase production and the amount of penicillin irreversibly and "specifically" bound, both being maximal at and above 1.0 u/ml (13). It is possible that the "nonspecifically" bound $S^a$, from the penicillin preparation is responsible, but there was no correlation between the amount of this and enzyme synthesis (the question of whether the "nonspecific" $S^a$ binding does represent penicillin is discussed in section I). Like other sensitive organisms, the concentration at which the penicillin irreversibly and specifically bound is maximal corresponds also with the minimal inhibitory concentration (1.0 u/ml with low inocula where little penicillinase is produced).

Thus, the specific PBC of the B. cereus is identical, as far as we can see, with that of S. aureus, with the exception of its connection with penicillinase adaptation. On the basis of the probability also discussed in section I that PBC in S. aureus is the site of action of penicillin, it seems likely that the PBC of the sensitive B. cereus also performs the same function because of this similarity. One is led to wonder how it is that inactivation of this component leads in one case only to death of the cell but in the other also provokes a vigorous protective reaction.

As Pollock (61) has pointed out, it is easy to speculate on the mechanism of penicillinase adaptation. One possibility, the simplicity of which merits consideration, however, is an analogy between penicillinase and diphtheria toxin. In Corynebacterium diphtheriae the continued metabolism of the cell in a deficiency of iron results in an accumulation of a substance which happens to be toxic to animals and which has been suggested to be bound normally in the iron-sufficient cell in the form of an iron-porphyrin complex (62). In B. cereus the analogue of iron would be the product $S$ of the normal function of PBC, and the analogue of diphtheria toxin would be the penicillinase-precursor (rather than penicillinase itself for reasons to be discussed in the next paragraph but one). The continued metabolism of a B. cereus culture in the absence of a given amount $Y$ of PBC per ml of culture (the result of binding a given amount $Y$ of penicillin) should result in the culture becoming relatively deficient in $S$ at a constant rate $kY$ proportional to the amount of PBC inactivated. There is evidence that inactivation of some PBC does not result in a stimulation of its rate of synthesis (18). Thus, if the rate of PBC function is normally geared to rate of production of some other substance (for example, so that $S$ can combine with it), this substance will be produced in excess at the same rate $kY$. This deficiency will become less important as it is diluted out by cell growth.

The rate of penicillinase production per ml of culture noted after "priming" with penicillin at 0 °C remained constant during growth and was proportional to the PBC fixed (13), so that the rate/g DW dropped by dilution with cell matter. This is in contrast to the exponential rate of
increase of many other adaptive enzymes. The PBC-radiopenicillin complex remained firmly bound to cell material throughout several generations (6, 13), so that the amount of S^{35}/g DW also dropped only by dilution with cell matter.

These facts prompted the suggestion that the PBC/penicillin complex was actually the catalyst concerned, which implies that PBC still retains some enzymic activity after binding with penicillin. The analogy with diphtheria toxin is simply the other alternative that binding with penicillin inactivates PBC completely, and appears simpler and is equally supported by the experimental evidence. Such an alternative implies that penicillinase is the substance which is normally united with the product of PBC, but this is not supported by the latent phase noticed between addition of penicillin and increase of rate of penicillinase synthesis (61). The latent phase, together with the "rebound" [transient increase in rate of synthesis (63)] and a shortening of the latent phase, both observed after a short period of anaerobiosis, has been satisfactorily accounted for by postulating the accumulation of a precursor whose synthesis was not affected by anaerobiosis, while the synthesis of penicillinase itself was stopped abruptly by the absence of oxygen.

The function of PBC is unaffected by anaerobiosis in S. aureus (18), and one may therefore suggest the following scheme for B. cereus:

```
facultatively
anaerobic
Earlier precursors ——> penicillinase precursor

facultatively
anaerobic
S precursor ——> PBC

S-(penicillinase-precursor) complex, a normal product retained by cell.
```

That penicillinase or its precursor plays a part in normal cell function is suggested by its occurrence in cultures grown in the absence of penicillin (59).

In the relative rates of production of PBC, S or penicillinase-precursor under anaerobic conditions or in the exposure of "reserve" PBC may lie a clue to the phenomenon of "reversion," where, if anaerobiosis is applied much before 40 min after addition of penicillin, the rate of synthesis of penicillinase on repassage of oxygen is less than before anaerobiosis. This scheme may also help us to understand why PBC and penicillinase, apparently unrelated substances, should both react with penicillin. Either S or its precursor (presumably sharing with penicillin some property such as acylating activity) can react with both PBC and penicillinase precursor, which is in turn likely to be very similar to penicillinase.

Whether or not one of these schemes is the correct one, it would appear that a study of the properties and normal functions of penicillinase and its precursors may well throw light on the initial reaction disorganized by penicillin. It would be interesting to see if the correlation between rate of penicillinase synthesis and amount of PBC bound extended even to the 'reserve' of PBC such as was found in S. aureus and which was only exposed during growth in the presence of penicillin (18). This might be achieved by adding penicillin continuously during growth at a rate greater than its destruction by penicillinase. Also the effect on penicillinase synthesis of inactivation of free PBC by other means, such as reaction with buffered acetyl chloride (8), should permit a decision between the two alternatives discussed above, i.e., as to whether simple inactivation of PBC or the presence of a penicillin "template" is necessary for penicillinase stimulation. It was mentioned in section IV that the action likely to be undergone by penicillin in binding PBC would change its chemical constitution so that it was no longer penicillin, but this does not necessarily mean that its molecular shape was so changed that it was incapable of acting as a template for penicillinase synthesis.

**Differences in Rate or Amount of Penicillin Binding**

An examination of the penicillin uptake over a relatively short time by resistant organisms which did not produce penicillinase and at
concentrations well below their minimal inhibitory level (8) revealed negligible binding of penicillin. The results of Maass and Johnson (5) were inconclusive with resistant organisms, some binding more penicillin than sensitive forms and some less. However, by increasing the penicillin concentration to equally effective (LD₉₀) levels, Eagle (15) found that the amount bound by naturally occurring resistant strains was relatively constant despite wide variations in their sensitivities. Thus it appears that in these cases penicillin resistance is quantitatively correlated with different "reactivities" of penicillin with PBC. This was not so with strains which had been selectively trained to resistance (12), where, in a manner similar to the experience of Maass and Johnson, the resistant strain could bind less or more or the same amount of penicillin as the parent sensitive strain. In one trial Eagle's strain of *Diplococcus pneumoniae* progressively bound less per gram over a period of about a year without change in sensitivity (12). This was also found with a staphylococcus by Cooper (18), and occurred simultaneously with an increase in cell volume so that the number of molecules bound per cell was roughly constant.

However, it is not clear whether the "reactivities" with penicillin of the strains described by Eagle truly refer to the final amount which is capable of being bound at a given concentration or to the rate at which binding occurs. If the cells were contacted for a longer time, the amount bound at subinhibitory concentrations might be significantly greater. It may be that the change in rate of binding with concentration is more important than the final amount bound in any correlation between "reactivity" and sensitivity. Maass and Johnson (6) and Cooper (19) have shown that the rate of penicillin binding increases with penicillin concentration over the inhibitory range up to a level where it becomes too rapid to measure. The low binding by Eagle's resistant strains at low concentrations, compared with sensitive strains, would lead to the expectation that at least in naturally resistant strains the rate of binding is considerably slowed, as the content of PBC and the rates of synthesis of PBC (rate of cell growth multiplied by content of PBC expressed as penicillin bound per gram when uptake is saturated) were all very similar. Any other effect of concentration would involve reversible binding.

The important question of rates rather than absolute amounts of binding does not appear to have been examined experimentally for resistant strains by any of the authors quoted, but it is interesting that the data of Reynolds et al. (64) and Pollock and Perret (13) show that the rates of penicillin uptake by two organisms that are relatively more resistant (*Streptococcus faecalis*, 8 u/ml, *Bacillus cereus*, 1 u/ml) are markedly slower at any given concentration than the rate of uptake on a sensitive *Staphylococcus aureus*, which is practically instantaneous above 0.1 u/ml. This has also been found with a staphylococcus just inhibited by 1 u/ml (Cooper, unpublished). There seem to be three different ways in which the rate of reaction of penicillin with PBC could be slowed in resistant strains: (a) the PBC itself may be less reactive chemically so that the proportion of effective to total contacts between penicillin and PBC becomes less; (b) steric hindrance becomes more marked, reducing the total number of contacts; (c) if the sensitive cell is in fact freely penetrated by penicillin, which cannot yet be decided, and if PBC resides internally, then resistance could be achieved by a decrease in the permeability of the cell wall to penicillin. This might come about for a variety of reasons, one of which could conceivably be a decrease in the proportion of lecithin in the cell wall phospholipids, as lecithin is penetrated by penicillin, while cephalin and cardiolipin are not (28). However, penicillin binding by sonic extracts of resistant cells is the same as that of intact cells, suggesting that there is no change in permeability but rather that the reactivity of the site is affected (12).

These ways taken together would permit an infinitely large possible number of small differences in penicillin resistance. They do not exhaust the list, however, for some strains may be able to synthesize PBC faster than others giving a high uptake in u/g DW, yet these would be more resistant to the drug, since their deficiency could be made good faster. Also, strains selected for resistance may have the same uptake in u/g as the parents, yet their rate of synthesis of PBC may be stimulated by penicillin or they may be less dependent on the product of PBC, making them apparently more resistant. On the other hand, the cell may alter in size, yet the amount of PBC required by each cell may remain constant, so that the uptake may vary without change in sensitivity.

In view of the rather confusing number of
theoretical possibilities it might perhaps be expected that little correlation between rate or amount of penicillin bound and sensitivity should exist. This somewhat pessimistic forecast renders all the more interesting the several observations that such a correlation does in fact occur in naturally occurring resistant strains, although not necessarily existing for strains selected for resistance.

**Apparent Independence of PBC**

If the cell does not possess PBC or is not affected by its loss, then the concept of PBC as the site of action of penicillin will mean that this cell will be resistant to penicillin. Most bacteria possessed PBC to some extent, but it seemed possible at one time that the penicillin-resistant yeasts may be independent of PBC, since they were incapable of binding penicillin (5, 19). More recently Daniel and Johnson at Wisconsin (16) showed that a soluble extract of ruptured yeast cells could bind penicillin in a specific manner similar to that of sensitive bacteria, so yeasts do in fact possess a PBC, but Cooper (19) found that, in contrast to staphylococci, the “lipid particles” of yeast obtained on mechanical rupture were incapable of binding penicillin. It is not known whether the specific binding of one yeast used by Daniel and Johnson is related to the lipid particles. Yeast cells were impermeable to penicillin (5), so that perhaps PBC is contained within the yeast cell and sheltered from penicillin rather than at or near the osmotic surface as in staphylococci.

Practically no “specific” binding of penicillin occurs with mammalian cells such as occurs with penicillin sensitive bacteria (32). It therefore seems likely that the lack of toxicity of penicillin for such cells is accounted for by their lack of PBC. It would be interesting, however, to see if penicillin is bound specifically by a small fraction of the cell material such as the subcellular particles (e.g., mitochondria). Such a small binding may be masked by the large amount of inert material present. The mitochondria are not freely permeable to all ions (65) and in fact seem to possess an osmotic barrier in some ways analogous to that of bacteria, so that it may be necessary to rupture them in order to allow the penicillin to penetrate. It would be interesting also to see whether the relative toxicity of penicillin for guinea pigs can be correlated with a specific type of fixation by any particular guinea pig tissue or subcellular particle.

If a cell is independent of PBC, then why should it be inhibited at all by penicillin at any concentration less than that which will interfere with the osmotic properties of the medium? The answer to this is probably that the type of inhibition exerted by penicillin changes qualitatively at higher concentrations, becoming bacteriostatic rather than bactericidal (17), suggesting that growth is inhibited by interference with difference sites in the cell. Thus, in comparing work on the “site of action” of penicillin one must bear in mind the concentrations of penicillin at which it was studied. Eagle (41) makes the interesting suggestion that the bactericidal action at lower concentrations could be due to accumulation of a toxic precursor, normally metabolized by PBC, so that the considerable slowing of killing at higher concentrations is due to slowing of the rate of synthesis of this precursor. However, as he pointed out, there may equally be a slowing of the rate of exhaustion of the cell’s limited reserve of metabolites by inhibition at higher drug titers of, e.g., nucleic acid synthesis. K⁺, Na⁺, Mg⁺⁺ or phosphate could fill the role of metabolites exhausted. Several metabolic effects of penicillin have been noted only at concentrations considerably higher than the minimal inhibitory titers (66, 67, 68, 69).

A reaction undergone by resting cells which is inhibited by high concentrations is glutamate exchange (66) and this is also 10 per cent inhibited at very low concentrations (0.01 u/ml). However, the inhibition is not complete even at 1000 u/ml. This inhibition only changes by 2 to 5 per cent over the minimal growth inhibition range of the test organism, in contrast to the sharp change in binding by PBC at about these concentrations, suggesting that this inhibition is not the result of fixation by PBC. The type of inhibition curve suggests some kind of reversible binding.

It could be inferred, from the observation that penicillin uptake does not increase in resting cells (18), that PBC does not turn over in the resting state, and therefore perhaps any reaction which can proceed in the resting state is unlikely to be the initial reaction disorganized by penicillin. If it is conceded that the increase of penicillin bound during growth does in fact represent turn-over of PBC, then in order to prove
As contact in the resting state binds only mechanisms of penicillin resistance have proved uptake on the effect on permeability to penicillin (64). Also, as contact in the resting state binds only 50% of the total PBC, then it would be expected that resting contact followed by washing should halve rather than eliminate the rate of the reaction under test on subsequent growth.

VIII. MECHANISM OF SYNERGISM

Two hypotheses have been considered likely to account for synergism between antibiotics (70): (a) one drug may increase the ability of the other to reach its receptor site, perhaps by increasing the permeability of the cell wall; (b) alternative metabolic pathways may be simultaneously blocked.

In the synergism which occurs between penicillin and streptomycin, possibility (a) above may result in an increased rate or final level of penicillin uptake if it is the streptomycin which has the effect on permeability to penicillin (64). Unfortunately, the rate and final level of penicillin uptake on Streptococcus faecalis seemed to be completely unaffected by streptomycin under resting or growing conditions, although synergism in the form of an increased rate of killing was clearly demonstrated when growth occurred. In view of the effects of penicillin on cell wall functions mentioned in section V, it would be interesting to try the reciprocal experiment of measuring streptomycin uptake in the presence of penicillin.

IX. CONCLUSION

Whether or not PBC is the site of action of penicillin and governs the reaction first disorganized it is evident that use of radiopenicillin appears to explain many previous experimental findings and may guide future ones. In general, the results of the four groups of workers concerned have agreed well, and illustrate the value of several independent approaches. Beyond that, the results up to the present are a little disappointing in that they represent only an introduction to the problem and often the actual happenings can only be inferred rather than proven. Radiopenicillin as a "rational approach" is only half the tool which is needed. The possible mechanisms of penicillin resistance have proved to be very complex; we are not sure how to approach the question of synergism, and the function of the reaction initially disorganized by penicillin and chemical nature of its site of action are still unknown. It can be seen that much further work needs to be done, and new techniques are needed in which radiopenicillin itself may play little part.

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A Method for Producing Plaques in Agar Suspensions of Animal Cells.

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A Method for Producing Plaques in Agar Suspensions of Animal Cells

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SUMMARY

A plaque method is described for animal viruses using agar-cell suspensions, which has certain advantages over the monolayer method.

INTRODUCTION

The plaque assay method for animal viruses (Dulbecco, 1952) involves adsorption of the virus on a monolayer culture of susceptible cells, after which an embryo extract-Earle’s saline agar medium is poured on and allowed to set. Plaques are visible when the cells are stained with neutral red after incubation of appropriate length. It has now been found that plaques can also be produced in an agar overlay cell suspension in a similar manner to the familiar procedure used for bacteriophage. This improves the efficiency of plating of both virus and infected cell suspensions and should give easier access to the versatile techniques developed for phage.

MATERIALS AND METHODS

Host cell-virus systems. The method was initially applied to vesicular stomatitis virus growing on chicken embryo cells. It has also been successfully used with the same virus on “L” cells (Earle), or with poliomyelitis virus type 1 growing on HeLa and monkey kidney cells (Vogt, personal communication). The following detailed description applies to the vesicular stomatitis-chicken embryo cell system.

Media. Earle’s saline containing 0.9% agar and either 12.5% embryo
extract or 0.5% enzymatic lactalbumin hydrolyzate (Nutritional Biochemicals Corp., Cleveland, Ohio) + 0.1% yeast extract (Difco-Bacto) + 0.1% crystalline bovine albumin (Armour, Chicago, Ill.: Youngner, personal communication) was used for both base layer and overlay. Replacement of bicarbonate buffer by 0.3% tris(hydroxymethyl)aminomethane (Sigma Chemical Co., St. Louis, Mo.: Dulbecco, personal communication) was also satisfactory for this particular system and made unnecessary the use of a CO₂-containing atmosphere during incubation.

**Method of plating.** (1) Three milliliters of a double-strength nutrient solution was added to 3 ml of molten 1.8% agar at 44°, and the contents were poured into flat-bottomed (pressed) 100-mm Pyrex petri dishes and allowed to set as base layers. (2) To 10-ml Pyrex tubes were added 0.5-ml samples of a cell suspension in Earle’s saline containing 1 to 1.5 × 10⁸ cells/ml which was freshly prepared from 10-day chick embryos (Dulbecco, 1952). Samples (0.1 ml) of a virus or infected-cell suspension containing 100 to 1000 plaque-forming particles/ml were added, followed by 2.0 ml of the molten agar medium at 44°. No adsorption period was found necessary. The cell-agar suspension was rapidly mixed, poured at once onto the base layer, and spread by rotating before it set. The tube was allowed to drain as completely as possible and the final drop removed by touching onto the agar surface. Less than 3% more plaques were obtained when the tubes were immediately rinsed with a fresh agar-cell suspension which was allowed to set on a separate base layer, so that drainage loss was negligible. (3) After setting, plates were incubated at 37° for 40 hours in a humid atmosphere (containing 4% CO₂ when bicarbonate media were used) before staining for 1 to 2 hours with Earle’s saline containing 1/20,000 neutral red, when the plaques could be counted.

**RESULTS**

**Plaque appearance.** Plaques in cell suspensions were similar in size to those in monolayer cultures in all three media but were less sharply defined. If plates were stained at 26 hours instead of 36 to 40 hours, the plaques were somewhat too small to be counted easily, and an additional 10 to 20% appeared if the stained plates were incubated for a further 14 hours.

**Plating efficiency.** The agar suspension method was developed initially to see whether substantially all cells were infected when a mono-
layer containing $2 \times 10^7$ cells had been allowed to adsorb $14 \times 10^7$ virus particles, i.e., to a presumed average multiplicity of 7. A monolayer infected in this way was washed four times, and the cells were removed with trypsin and washed four times again. After the large clumps had settled for 2 minutes, the supernatant was removed for counting in a hemocytometer and for serial dilution in Earle's saline-4% ox serum which contained $10^7$ noninfected cells/ml to minimize cell death and adsorption by glassware. Silicone-treated glassware was also used. Samples of the diluted cells were plated as described above while still in the latent period. Plaques were formed by 62 to 75% of the cells which could be counted. This compared with 20 to 40% by the monolayer method, presumably due to the low efficiency of adhesion of the cells to the monolayer in the 30 to 60 minutes before agar was added. The supernatant from the original undiluted suspension was not separated from the cells until the diluted samples had been plated, and then it accounted for only 0.06% of the plaques found, so that free virus was negligible.

When assaying free virus, the cell suspension method in all media gave 30 to 50% more plaques than the monolayer method in parallel assays from the same virus dilution. Plating efficiencies did not differ by more than 10% when the three media were compared by either method alone.

Viability of cells. It appears to be essential that at least 90% of the population stain with neutral red after incubation is complete. Death of cells either before or during incubation markedly lowered the plating efficiency and rendered the plaques indistinct and difficult to count. For this reason the agar overlay should contain more than $1.5 \times 10^7$ cells/ml initially. It is probable that no cell multiplication occurred. After 2 days approximately 80% of cells kept at this concentration stained with neutral red, but below this level many fewer cells stained and the plating efficiency was less. Very few cells stained and no plaques were visible after 2 days at $0.5 \times 10^7$ cells/ml, whereas the plating efficiency was constant between $1.5$ and $3.0 \times 10^7$ cells/ml. A concentration of 2.0 to $2.5 \times 10^7$ cells/ml was usually employed. It was possible to store chick cells overnight at 4° in Earle's saline-4% ox serum ($2 \times 10^5$ cells/ml) without clumping and still obtain good plaques with a plating efficiency 20% greater than the monolayer. However, poor results were obtained if cells were kept for a few hours at $10^8$ per milliliter in a warm room, possibly owing to anoxia.
Plating of virus on preset agar-cell suspensions. As with bacteriophage, plaques can be obtained by adding virus to agar-cell suspensions after they have been allowed to set. For this purpose it is preferable to use an agar concentration of 1.2% for both layers, to dry the plate in an open and inverted position for 30 minutes at 37° before adding virus, and to use a glass spreader rather than a wire loop. Figure 1 shows that individual plaques were separated by spreading in a similar manner to colonies of bacteria. Such plates may be convenient for surveying large numbers of samples where the initial titer is not known, and in clinical applications they may reveal mixed viral populations not shown by mass culture methods. They can be stored at 4° for at least 2 days before use. A spot test can be made by placing loopfuls of virus dilutions on the plates. This is in effect an end-point method suitable for rough assay of a large number of samples such as may be obtained in genetic experiments. A similar method has already been used by Dulbecco and Vogt (unpublished) with poliomyelitis virus on preset agar-cell monolayers. A less readily diffusible (larger) or less stable virus may require the shorter diffusion path provided by the cell suspension method.

Uses and limitations. The advantages of the agar-cell suspension include (1) no contact between cell and glass, so that toxic effects of glassware should be less; (2) possible application to any cells not forming monolayers; (3) easy use of mixed cell populations (e.g., to produce “turbid” plaques); (4) higher plating efficiency for free virus and in-

Fig. 1. Vesicular stomatitis virus suspension (original titre 9 X 10⁶ plaque-forming particles/ml) spread in counter-clockwise direction on agar-cell suspension plate to give single plaques (X 1).
fected cells; (5) no time interval required for adsorption; (6) individual plaques separable from high titer suspensions by spreading; (7) use of a spot test for large or unstable viruses. Two disadvantages have been found: (1) chick cells will not all survive below a concentration of $1.5 \times 10^7$/ml in an agar suspension; (2) a given number of embryos yield 2.5 times as many monolayers as suspension plates. These limitations may not apply to other cell systems.

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Some Characteristics of Vesicular Stomatitis Virus Growth-Curves in Tissue Culture

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SUMMARY: Vesicular stomatitis virus was released exponentially and fairly reproducibly after infecting chick embryo cell monolayers. This system appeared suitable for chemical studies, which are to be presented separately; the present paper is concerned with growth-curve methods and a discussion of the system. When the average multiplicity of adsorption was 6 plaque-forming units/cell the latent period was 1–2 hr., and growth appeared to follow a one-step cycle. At least 62–75% of the cells were capable of releasing virus.

This paper is intended to show that vesicular stomatitis (VS) virus growing in chick embryo-cell monolayers is a suitable system for quantitative study. It describes the conditions used in such studies, the results of which are to be presented separately (Cooper, 1957a, b).

Intracellular chemical changes during infection should give information on virus growth, but a suitable system needs the following properties: (a) virus should be assayed by particle count methods and should be released into a fluid medium and not extracted in unknown yield from whole tissue; (b) one-step growth conditions must apply, so that changes take place in all cells together as much as possible (the most informative changes occur during the latent or exponential release periods which must be identifiable); (c) nearly all cells must be infected at zero time, so that pathological changes are not masked by metabolism of healthy cells; (d) samples must be representative and reproducible; (e) cells should produce virus at an optimal rate, so that changes are maximal. These criteria are discussed below in relation to the system used. It is unfortunate that technical difficulties have hindered interpretation of many experiments with animal viruses (Bauer, 1952), because at least one of these properties was missing.

METHODS

General type of experiment. Most experiments involved harvesting one virus-infected and one control monolayer of embryo cells and the entire supernatant fluids after varying times during incubation at 37°C. A group of monolayers all from the same batch were infected nearly simultaneously as described below, and a growth curve was obtained by plotting the total plaque-forming units (pfu) in the completely removed supernatant fluids against the time of harvesting. Thus six points on a growth curve would require six monolayers; successive samples from the same plate were not used. The method for harvesting the cells is given with the chemical methods (Cooper, 1957a).

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Virus growth and phosphorus metabolism should be similar in cells prepared identically from chick embryos of the same age, and the state of any one monolayer and supernatant at a given time should represent that of all others from the same batch when treated identically. That these assumptions are justified is supported by: (a) the reproducibility of the growth curves discussed below; (b) the fairly smooth and reproducible nature of the $^{32}$P uptake curves (Cooper, 1957a) and the low standard deviations of the total $^{32}$P taken up by two sets of 10 identical monolayers (about 5% of the mean uptakes).

**Virus stocks.** The virus used was a New Jersey (Missouri) strain of vesicular stomatitis virus kindly provided by Dr S. Madin, which had been shown to make good plaques on chick cells by Sellers (1955 and personal communication). All stocks were derived by diluted inoculum passage from a master culture, which was the 10 hr. supernatant fluid (5 ml.) from a chick embryo monolayer infected with a suspension of a single well-separated plaque.

The presence of a transmissible interfering component ('incomplete virus?') in some vesicular stomatitis virus stocks is to be reported elsewhere. All experiments described here and by Cooper (1957a, b) used stocks which are regarded as containing very little of this component, as they had high titres and were made by not more than three diluted passages from the original presumed single particle. Virus yields (pfu/cell) during experiments were high.

Stocks were kept at $-20^\circ$ for not more than 3 months; the titre was generally $2 \times 10^9$ pfu/ml., but some inactivation was detected on storage. Each cycle of freezing and thawing in Earle's saline + 4% (v/v) serum resulted in an inactivation of about 15% of the plaque-forming units.

**Virus assay.** The plaque method used was that described by Rubin, Baluda & Hotchin (1955) for Western Equine Encephalomyelitis virus and cells from 10-day chick embryos, except that embryo extract in the agar overlay was replaced by 5 mg./ml. lactalbumin hydrolysate + 1 mg./ml. yeast extract + 1 mg./ml. crystalline bovine plasma albumin (Dr J. S. Youngner, personal communication). The curves presented are relative assays not corrected for plating efficiency, and amounts of virus are described in terms of plaque-forming units in the assay system used. Growth and storage took place entirely in darkness, and during virus assays virus was in serum-free media for less than 5 min. under exclusively artificial (fluorescent strip) lighting, so that light inactivation (Skinner & Bradish, 1954) was negligible. One plaque results from a single 'infective centre' (Franklin, personal communication); the 'infective centre' may be a clump of virus particles but virus preparations gave single-hit X-ray and UV inactivation curves and were therefore presumed to be monodisperse, so that one plaque results from one infective virus particle. The average multiplicity of infection was determined from the number of pfu adsorbed by the monolayer and the number of cells present in the monolayer. The number of cells was found by haemocytometer counts of a trypsin re-suspension. The number of pfu adsorbed to individual cells was assumed to follow a Poisson distribution. Whether all the particles adsorbed to a cell or only a fraction thereof participate in viral reproduction within that cell is at present unknown.
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*Media.* Earle's saline (1948) containing 4% (v/v) horse or more often ox serum (ES/Ox) was added to infected or control monolayers for the period of virus release; no difference was found between sera provided that antiviral activity was absent. Serum was included for its small stimulation of the rate of $^{32}$P uptake and better effect on appearance of cells, particularly after 20 hr. Virus was diluted for assay in phosphate buffer saline (PBS, Dulbecco & Vogt, 1954a).

*Preparation of monolayers of chick embryo cells.* Monolayers were prepared for growth-curves and for routine plaque-assays by the same method. This was either the pulping procedure described by Rubin *et al.* (1955), or else the embryos were coarsely minced and digested by gentle agitation in trypsin (Melnick, 1955). The latter method gives three to four times as many cells as the former and the performance of the monolayers appears to be identical. Monolayers were always used for growth-curves (and usually for assays) after 20 hr. incubation when cells had spread to form a continuous sheet. The $^{32}$P uptake continued at a constant rate for the first 2 days but had nearly ceased by the third day.

*Method employed for one-step growth-curves.* To infect the cells, medium was removed from 20 hr.-monolayers which were then washed once (or three times when containing $^{32}$P) with 5 ml. ES/Ox. After washing, 0.5 ml. of ES/Ox containing virus at known titre (or without virus for controls) was added, and allowed to spread over the whole surface (2-3 min.), when the plates were incubated at $37^\circ$ in a humid atmosphere containing approximately 5% CO$_2$ for 30 min. Addition of 4.5 ml. of warm ES/Ox was followed by washing in some experiments, but where washing is not mentioned the medium was allowed to remain on the plates at $37^\circ$ until entirely removed at different times and frozen pending virus assay. Unless otherwise mentioned, the inoculum contained 7.5 pfu for each cell in the monolayer, and assay of the medium shortly after making up to 5 ml. showed that adsorption was 60-90% complete (average 80%) giving a final average multiplicity of adsorption of 5-6. Little further VS virus was adsorbed by 10 cm. Petri dish monolayers after 30 min. with 0.5 ml. of inoculum, and an average multiplicity of 1 was probably reached 10-15 min. after its addition. The time of first addition of virus was regarded as zero, so that the latent periods include the period required for the virus to come into contact with the cells.

**RESULTS**

*Establishment of the time required for a presumed one-step growth-curve* Fig. 1 shows the virus release-time relationships obtained from a number of experiments using the growth-curve method described above. Duplicates agreed well, and fairly smooth curves were obtained. The latent period is defined throughout the present work as the average time required for each cell to release one pfu; this is regarded as the least ambiguous way of defining the rate of development of a system in which no sharply delineated ‘burst’ occurs. The latent period is determined when necessary by extrapolating the
Fig. 1. Total number of plaque-forming units released by chick embryo cell monolayers (2 x 10^7 cells) infected to an average multiplicity of adsorption of 6 with vesicular stomatitis virus. All points of each curve were obtained with the same batch of plates, but except where mentioned the complete curves were made on separate occasions with different batches of plates. Two experiments (O—O, washed once; ●—●, washed three times) are given to show the extreme range in latent period encountered among twelve experiments; in all the experiments virus release increased exponentially at similar rates. Variations between duplicates are shown by (●—●); (△—△) was washed once and (△—△) was washed three times with medium before infection, but were otherwise identical and from the same batch of plates. Two curves (●—●) and (○—○) were infected with multiplicity 1–2 (uncorrected for cells theoretically uninfected) but otherwise were identical with those of multiplicity = 6. The bar at 10 hr. indicates the range of the yields from seven different multiplicities of adsorption varying from 0.5 to 23. The point on the ordinate represents the virus added as inoculum for multiplicity = 6, and the middle broken horizontal line the average amount of virus left unadsorbed after 30 min.; the lowest broken line indicates the level of termination of the latent period, and the uppermost broken line the average 20 hr. yield from once-washed monolayers.
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exponential rise in a logarithmic plot back to 1 pfu/cell. The average latent period was 1.5 hr. at a multiplicity of 6; all latent periods at this multiplicity fell within the range of 1.0–2.0 hr. Exponential release of 50% of the final yield occurred up to 6 hr., and the remainder was released more slowly for some hours further. Although the latent periods varied somewhat between experiments, the rates of virus release were closely similar, and except as described the final yields were also similar; the threefold washing of the plates before infection required by some experiments gave a considerably lower yield than one wash. The small differences in latent period may be due to factors (such as small variations between batches of plates) other than differences in washing of the plates, as one experiment with the same batch of plates where the washings differed gave similar latent periods although slightly different rates of release and different final yield.

Proportion of cells supporting viral growth

Adsorption of $12 \times 10^7$ pfu by a uniform confluent monolayer of $2 \times 10^7$ cells should be sufficient to place virus on 99.7% of the cells. That most cells with adsorbed virus are capable of supporting growth is regarded as proved by the following observations: (a) 40 hr. plaques on chick embryo monolayers show a fairly sharp boundary within which no live cells are visible, so that none of the population is completely resistant; (b) as described elsewhere (Cooper, 1955), when cells were removed during the latent period with 0.25% trypsin from monolayers infected to multiplicity of 6–7, 62–75% of the cells counted in a haemocytometer were able to produce plaques when mixed in an agar cell suspension containing $2 \times 10^7$ non-infected cells/ml. All cells visible were included in the count, even those apparently in poor condition, so that the considerable chance of cell trauma during their treatment means that this figure of 62–75% is certainly appreciably low. Evidence was presented by Dulbecco & Vogt (1954a) to show that cells in a monkey kidney monolayer were equally susceptible to poliovirus.

Effect of multiplicity of adsorption

Fig. 1 shows that the two release curves with multiplicity 1 to 2 had a longer latent period than those with multiplicity 6, while the rate of release and 10 hr. yield were independent of multiplicity. These effects will be discussed more extensively elsewhere. Meanwhile the presentation of these experiments is anticipated in Fig. 2, as the effect of multiplicity of adsorption on latent period has some important inferences for chemical studies.

As cells probably adsorb virus according to a Poisson distribution, the multiplicity of individual cells in an infected monolayer will vary. The effect of multiplicity of adsorption on latent period will mean therefore that at any one time the cells in such a monolayer will be at different stages of their growth cycle. This is a characteristic undesirable for chemical study but one which appears inevitable if one insists that all cells are infected initially. To estimate the extent of the discrepancies, theoretical release curves were constructed for an ideal monolayer randomly infected to an average multiplicity of 6 by
applying the observed relationships between multiplicity and latent period, and assuming that cells at different multiplicities release virus at the same exponential rate and to the same yield, even though starting at different times. It is further assumed that all cells of the same multiplicity start together, and

![Diagram showing theoretical curves for virus release](image)

**Fig. 2.** Theoretical curves attempting to evaluate the differences in virus release to be expected from a Poisson distribution of multiplicity of infection in a monolayer infected to an average multiplicity of 6 (multiplicities higher than 12 are ignored). The commencement of each curve corresponds with release of one pfu for the average cell at that multiplicity, and is calculated from the latent periods of monolayers infected to that multiplicity as an average; only seven of the twelve curves are given.

the later non-exponential release is ignored. Some illustrative curves are given in Fig. 2. From these curves it can be calculated that 80% of the cells are less than 0.7 hr. apart in starting or completing their release, 60% < 0.4 hr. apart and 30% < 0.1 hr. apart; a negligible proportion of cells complete their release more than 0.6 hr. before the mean time for completion. As samples are
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usually taken 1 hr. apart this probably means that virus growth will be sufficiently synchronized for any chemical change due to virus growth to be the major one, and not to be too obscured by healthy metabolism of late-starting cells or lysis of early starters. Nevertheless, the fact that these curves represent only an average would have to be borne in mind in, for example, placing any small change in its correct time sequence with regard to virus growth. The theoretical curves do not, of course, consider cells which are early or late starters for reasons other than multiplicity, and some further spread will be introduced by the non-simultaneous infection of the cells due to the 30 min. adsorption period required.

**DISCUSSION**

That the experiments described above represent one-step growth cycles is demonstrated by: (a) relatively rapid adsorption of sufficient plaque-forming units to infect all cells initially; (b) release of virus by most infected cells; (c) the release of 50% of virus within the exponential period, without previous or subsequent steps; (d) the similarity of other animal virus growth systems likely to be one-step curves (Dulbecco, 1955); (e) the almost simultaneous cessation of $^{32}$P uptake by many fractions without concurrent lysis during the exponential period (Cooper, 1957a), indicating that most cells are affected at this time.

Continued slow non-exponential release may be a function of delayed cell death, as by 6 hr. less than 25% of the cells were ‘rounded-up’ and release of phosphorus from most cellular fractions was very small (Cooper, 1957a). Thus non-specific lysis at this stage must also be small. Most cells were rounded and lysis was extensive by 20 hr.

It is a disadvantage of this system that the cell population is not homogeneous and can be seen to contain fibroblast and epithelial type cells. However, fibroblasts are in considerable excess, and a mixture probably does not matter for the present purpose, as McClain & Hackett (1955) showed that latent and release periods of VS virus were closely similar in three epithelial and two fibroblast cultures from five different vertebrate species; of these systems chick cells produced most virus and therefore may show most changes. The intact monolayer was preferred for virus growth to the cell suspension method for this reason, as the latter often gave lower yields per cell (see also Dulbecco & Vogt, 1954b).

It thus appears that most of the criteria listed in the introduction as necessary for chemical studies are fulfilled by this system.

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Paths of Phosphate Transfer in Normal Chick Embryo Cells and in Cells Infected with Vesicular Stomatitis Virus

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SUMMARY: The paths of phosphate transfer were compared in normal and vesicular stomatitis virus-infected chick embryo cell monolayers during one-step virus growth. Phosphate entered the normal cell via an inorganic pool in reversible equilibrium with external phosphate, and passed to large-molecule phosphate from this pool or from organic acid-soluble phosphate. During the latent and exponential release periods of virus infection there was no detectable slowing or stimulation of the rate of gain of $^{32}$P by acid-soluble inorganic and organic phosphates (AI and AO), lipid phosphate (LP), ribonucleic acid (RNA) and other phosphate fractions until uptake ceased in nearly all fractions about half-way through exponential release. Negligible P or $^{32}$P entered or left deoxyribonucleic acid (DNA) in normal or infected cells in this system. Before and during exponential release there was no detectable lysis of nuclei, mitochondria or microsomes (examined after isolation), and no detectable loss of $^{32}$P from AI, AO, LP, RNA or DNA, except for a late 30–50 % decrease in the $^{32}$P of the sucrose-soluble RNA of disrupted cells. This could be a secondary effect (i.e. onset of cell death) rather than an essential stage of virus growth. Gross lysis was evident in all fractions 20 hr. after infection, with the exception of the acid-soluble inorganic fraction not in reversible equilibrium with the medium.

Viral nucleic acid synthesis should be reflected in some changes in phosphate metabolism. A study of such changes should provide information on virus growth complementary to direct examination of infected cells (e.g. by the electron microscope) which is likely to show stages of maturation rather than of nucleic acid replication. Chemical changes should tell more than relative assays of infective unit content when examining subcellular fractions, as such assays only count subcellular particles with attached mature virus rather than the number or nature of virus attached; non-adsorption of released mature virus to free subcellular particles may not be a good control. Furthermore, this overlooks possible viral replication in the ‘soluble’ cytoplasmic portion, as infective virus, being particulate, will always be removed from this fraction.

The vesicular stomatitis (VS) virus-chick embryo cell system can satisfy certain criteria for chemical study of the effects of virus growth (Cooper, 1957a). Such a study is presented below, where phosphate metabolism is compared in normal and infected cells during the latent and exponential release periods.

There is little data on normal phosphate pathways available for intact animal cells in vitro with all cells equally accessible to the medium, and the chick cell system, although definitely limited metabolically, has some tech-

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technical advantages for such a study over the living animal. In particular, one
knows the specific activity of the external phosphate, and one can trace
phosphate pathways from the specific activities of increments of homogeneous
phosphate fractions (Mitchell & Moyle, 1953). Some information on the entry
of inorganic phosphate is presented separately (Cooper, 1957b).

METHODS

Preparation of chick cell monolayers, virus stocks, virus assay, media and the
method for the one-step growth curve on monolayers are described by Cooper
(1957a).

Type of experiments described. In most experiments, one infected and one
control monolayer plus media were harvested at intervals to give one-step
growth curves lasting less than 24 hr. In the first 2–3 days, non-infected
monolayers lost negligible amounts of soluble or particulate RNA or DNA,
asimilated $^{32}$P maximally, remained normal microscopically and grew virus
well so that negligible cell material was lost or changed by ‘non-specific’
death, lysis or detachment.

Methods of studying $P$ pathways. The words ‘exchange’, ‘gain’ or ‘loss’ are
used to define the phosphate transfers, rather than the ambiguous ‘turnover’.
Three types of experiment were used.

(a) ‘Specific activities of increments.’ The relative specific activities (‘$R$
values’), i.e. the ratios of the observed values of $^{32}$P/$P$
to that value to be
expected at complete equilibration (Mitchell & Moyle, 1953), of some Schmidt-
Thannhauser fractions were determined at intervals during uptake of $^{32}$P by
cells. Then, if a fraction $A$ is homogeneous the specific activity of any small
sample passing from $A$ into another fraction $B$ (‘specific activity of the incre­
ment of $B$’) will have the specific activity of $A$ (Mitchell & Moyle, 1953). Thus,
knowing the specific activity of the increment of $B$, the true precursor pool to
$B$ is known if the only alternatives have sufficiently different specific activities.
In practice this method is limited, since few pools are homogeneous and a
single specific activity of an increment rests on four assays so that errors are
magnified. It was only used for normal pathways.

(b) ‘Gain’ experiments. $^{32}$PO$_4$ was added with the virus (i.e. to monolayers
20 hr. old) and the increase with time of the $^{32}$P of each fraction was followed
in control and infected cells during one-step virus growth.

(c) ‘Loss’ experiments. $^{32}$PO$_4$, added to the Petri dishes with the cell
suspensions, was incorporated overnight at $37^\circ$. After 20 hr. the medium was
removed, the cell layers washed three times with warm medium, virus added
for one-step growth and the progressive loss of the total $^{32}$P of each fraction
was followed. The $^{32}$P added did not exceed $1\mu$C/ml. medium.

Cells were fractionated after ‘gain’ and ‘loss’ experiments by the Schmidt-
Thannhauser, sucrose and sucrose $+$ CaCl$_2$ cell homogenization, and citric acid
methods described below. The two complementary experiments proved quite
informative for $P$ pathways in control cells, particularly for differentiating
those fractions which were in reciprocal exchange from those which were not.
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They should also show virus-induced lysis, or stimulation of incorporation or exchange, which if big enough should affect the total $^{32}$P of any fraction in either or both 'gain' or 'loss'.

Harvesting of cells. The medium was frozen at intervals for virus, P or $^{32}$P assay, and the cells resuspended from the unwashed monolayer with 5 ml. 25 mg./ml. trypsin (10 min. at 37°) were quantitatively removed, washed once with 5 ml. cold phosphate buffer saline (PBS) and frozen for Schmidt-Thannhauser separation, or washed once with 5 ml. cold 0·25M-sucrose for homogenization or citric acid extraction.

Sucrose homogenization. The method of Hogeboom, Schneider & Striebich (1953) was used. The washed cells were quantitatively transferred to a Potter-Elvehjem glass homogenizer in sufficient (0·15 ml.) 0·25M-sucrose to fill the space between pestle and container without overflowing and without air bubbles. The pestle was rotated by an electric motor at an arbitrary constant speed while the container was held in a cradle; the tension was kept constant for all samples by supporting the cradle with stretched rubber bands. Each sample contained $2 \times 10^7$ cells, and the cells were always kept at ice-water temperature. The rotation time (usually 60 sec.) allowed at least 95% cell rupture (haemocytometer count). Recovery of nuclei, counted in 0·1M-citric acid containing 1 mg./ml. crystal violet, was 75–90% in sucrose alone, but after addition of 0·0018M-CaCl$_2$ according to Hogeboom et al. (1953) was always more than 95%. The ruptured suspension was quantitatively removed and centrifuged for 10 min. at 1500 rev./min. to sediment remaining cells and nuclei, either over a 1 ml. layer of 0·34M-sucrose (giving excellent separation) or directly, and washed once with 2 ml. 0·25M-sucrose. The pooled supernatant fluids were centrifuged for 10 min. at 10,000 rev./min. in the Spinco rotor No. 40, and the opaque sediment ('mitochondria') washed once with 2 ml. 0·25M-sucrose. The supernatant fluids, again pooled, were then centrifuged at 25,000 rev./min. for 1 hr., and the almost clear supernatant removed ('soluble fraction'). The translucent pellet ('microsomes') was resuspended in 1 ml. 0·25M-sucrose without washing. The contamination in the nuclear fraction (calculated from theoretical dilution by washing) was <3% of the cytoplasm using sucrose alone, but 80% of its radioactivity may be due to the 5% of cells remaining unbroken (better preparations were not possible because of the relative fragility of nuclei). The addition of 0·0018M-CaCl$_2$ to the sucrose, however, gave very good recoveries of nuclei substantially uncontaminated with whole cells, but in this case mitochondria were not separable from microsomes. Therefore, similar experiments were performed in presence or absence of CaCl$_2$ to examine particularly either nuclei, or mitochondria plus microsomes respectively. The mitochondrial fraction contained <0·5% (v/v) of the microsome + soluble fractions, and the microsomes 2–4% (v/v) of the soluble fraction.

Citric acid fractionation. This method (Sanford, Earle, Evans, Waltz & Shannon, 1951) was as follows. To the pellet of sucrose-washed cells 2 ml. of 0·1M-citric acid were added and the tubes immersed in a 37° water bath with periodic vigorous pipetting. After 1 hr. the tubes were centrifuged for 10 min. at

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1500 rev./min., the supernatant fluid removed and 2 ml. fresh citric acid added. Then after 1 hr. at 37° the tubes were kept at 2° overnight, centrifuged, the supernatant fluids pooled ('citric acid soluble' fraction) and the sediments ('nuclei') resuspended in citric acid for assay. Recovery of nuclei (haemocytometer count with 0-1 % crystal violet) was 80 %, intact cells were < 2 %.

*Modified Schmidt-Thannhauser separation method.* This was developed from Mitchell & Moyle (1953), Davidson, Frazer & Hutchison (1949), Davidson & Smellie (1952) and Katchman & Fetty (1955), and is presented schematically in the diagram. Some abbreviations and definitions, mostly derived from Mitchell & Moyle (1953) are:

- 'Hot acid hydrolysis' (Katchman & Fetty, 1955) = addition of 1/10 vol. 10n-HCl, immersed in boiling water for 15 min.
- 'Mg pptn.' = addition to 1 ml. sample of 0-1 ml. 5 % MgCl₂ + 0-1 ml. 0-2 m-phosphate (as carrier) + 0-1 ml. conc. ammonia solution (sp.gr. 0-880). After 16 hr. at 2°, the crystals were sedimented in the centrifuge.
- 'Acid-soluble' = soluble after 30 min. treatment with 10 % (w/v) trichloroacetic acid (TCA) at 2°.
- 'mI' = inorganic phosphate of the medium.
- 'mAO' = organic acid-soluble phosphates present in medium but derived from cells.
- The remaining fractions are defined as being present in the cell only.
  - 'AI' = acid-soluble inorganic orthophosphate.
  - 'AO' = acid-soluble organic phosphate stable to 'hot acid hydrolysis'.
  - '7P' = acid-soluble phosphate converted to AI by 'hot acid hydrolysis' ('seven-minute phosphate', Katchman & Fetty, 1955).
  - 'LMP' = acid-insoluble phosphate (large molecule phosphate).
  - 'LP' = hot-methanol-soluble phosphate (lipid phosphate).
  - 'RNA' = acid-insoluble phosphate transformed to AO by warm alkali, and regarded as predominantly derived from ribonucleic acid phosphate.
  - 'PP' = acid-insoluble phosphate transformed to AI by warm alkali, and including phospho-protein phosphate.
  - 'LM7P' = acid-insoluble phosphate transformed to '7P' by warm alkali.
  - 'DNA' = essentially residual phosphate, mostly derived from deoxyribonucleic acid phosphate, but containing some residual RNA and PP phosphates.

In one 'gain' experiment (Fig. 2c) RNA was extracted from the cells after harvesting by hot 10 % (w/v) NaCl according to Davidson & Smellie (1952). Extract and residue were subsequently fractionated by the Schmidt-Thannhauser procedure.

*Efficiency of the fractionation procedures.* It is acknowledged (e.g. Hogeboom et al. 1953) that the current methods of fractionating cells into their particulate components are unsatisfactory, and it is clear from the above procedures that, for example, 'mitochondria' will contain a small proportion of microsomes and nuclei, by their aggregation or disintegration, even if each fraction be completely homogeneous with respect to particle size. 'Mitochondrial' and 'microsomal' preparations have been observed to contain at least two
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(Laird, Nygaard, Ris & Barton, 1953) or three (Novikoff, Podber, Ryan & Noc, 1958) types of components, respectively, and the 'sucrose-soluble' and citric-acid soluble fractions will clearly contain any extractable substances which properly belong to a particulate fraction. The present work is therefore

20-30 mg. wet wt. cells (2 x 10⁷ cells) + 2 ml. cold 10% trichloroacetic acid (TCA) for 30 min., + 2 x 2 ml. TCA washes

Acid-soluble = Al + AO + 7P

Mg pptn. Hot acid hydrolysis + Mg pptn.

Soluble Insoluble

AO + 7P AI

Acid insoluble = LMP

One wash with 2 ml. cold methanol (MeOH), extract 2 x 5 ml. MeOH in 70° bath for 15 min., dry residue

MeOH-soluble = LP

Evaporate dry and digest conc. HClO₄ 16 hr. at 125°

Add 1 ml. n-NaOH, keep at 37° for 16 hr. (all apparently dissolves), cool in ice, add 1 ml. TCA + 0.1 ml. 10n-HCl, after 30 min. at 0° centrifuge and wash 2 x TCA

Soluble = RNA + LM7P + PP)

Mg pptn. Hot acid hydrolysis + Mg pptn.

Soluble = RNA + LM7P = PP + LM7P

Insoluble = DNA + some RNA + PP

Extract 2 x 2 ml. TCA at 100°

Scheme of modified Schmidt-Thannhauser separation method. The names of the fractions (e.g., 'RNA') are used by virtue of other workers' results with this method, and serve as definitions rather than as indications that these fractions have been checked chemically by the author.

Estimation of P and ³²P. Phosphate was determined as inorganic phosphate after 10 hr. digestion at 120° with 0.5 ml. HClO₄ by the method of Fiske & SubBarow (1925). ³²P was determined after drying the neutralized samples on 1 cm. copper planchettes by means of a Nuclear Instrument and Chemical Corp. (Chicago) automatic end-window Geiger counter with window thickness c. 0.5 mg./cm.². All samples were counted for at least 1000 counts and correction was made where necessary for radioactive decay and background counts.

obliged to define the fractions obtained solely in terms of their mode of preparation. Except for the nuclei, whose purity can be partly observed, much reserve must be attached to interpreting the fractions in terms of their function in the intact cell.
Preparation of fully-labelled cells. Twenty-five fertile eggs were inoculated via the yolk sac with 20 μc. of 32P in 0.2 ml. of Earle's saline (ES) before incubation. Only 6 embryos survived to 10 days, while the remainder were bacteriologically sterile but only 1 cm. long (fertility rate of un-inoculated eggs was 80%). At 10 days the full-size embryos were removed, cells prepared and allowed to form as monolayers overnight in the usual way in non-radioactive medium. After 20 hr. the monolayers were washed twice with medium, and the usual one-step growth 'loss' experiment performed, the cells being harvested and fractionated by the Schmidt-Thannhauser procedure. Virus production was, if anything, somewhat higher than average.

RESULTS

Normal pathways of phosphate transfer in monolayers 20–40 hr. old

At least five main chemical fractions appeared to exist in each of at least four main subcellular fractions. Few, if any, of the fractions were homogeneous. An attempt is made to trace the broad phosphate pathways among the major chemical (Schmidt-Thannhauser) fractions in the cell as a whole, and from P and 32P contents of centrifugal fractions at particular times certain further details may be deduced. The interpretations summarized in Fig. 1 are given at this stage to help presentation of the evidence.

Source and function of AI. 32PO4 passed from the medium into AI much faster than into other fractions, while no net increase of the AIP occurred (Table 1); the transfer had largely ceased after 4–5 hr. (Fig. 2) when the specific activities of AI and mI were substantially equal (Table 1). The implied equal loss of non-radioactive P should be large and rapid and was not found as a gain in any organic fraction, but the reverse loss of AI 32P back to mI occupied the same period of time (Fig. 3) so that evidently at least some of AI is in reversible equilibrium with mI. However, only 30% (Table 3) to 50% (Fig. 3) of the AI 32P gained in overnight incubation, and equivalent to about half the AIP of the cells (from Tables 1 and 2a) equilibrates in this way, showing that AI is not homogeneous. The two AI fractions can be called ‘reversible’ (AI1) and ‘irreversible’ (AI2) and appear from these data to have similar specific activities. AI can only be distinguished from AIK as 32P after equilibration with an excess of non-radioactive mIP, when (Fig. 3 and Table 3a) the AI, 32P was twice the highest value of the AO 32P, while the total AI, P must be considerably less than the total AOP (from Table 1, assuming that AI, P ≤ half [AIK + AI2]). This large difference in specific activity between AO and AI only allows that AI1 is rapidly derived from AIK or mI at a rate similar to the exchange into AIK, and not from AO or large molecule phosphate (LMP). Its stability in the 'loss' experiments, even after 20 hr., suggests that it is not an intermediary in P metabolism. It may be a very acid-labile polyphosphate; its stability to the presumably enzymic lysis in infected cells up to 20 hr. (Fig. 3) does not suggest an organic phosphate.
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Fig. 1. Diagram of suggested phosphate pathways. Evidence for the restrictive osmotic barrier is given elsewhere (Cooper, 1957b). Figures represent distribution of $^{32}$P after a 'loss' experiment of 7 hr. (data from Table 3a) calculated as percentage of total $^{32}$P in washed cells at commencement of 'loss'.

Fig. 2. A $^{32}$P 'gain' experiment (a and b) followed by Schmidt-Thannhauser (S-T) fractionation of whole cells. Circles represent control, and triangles infected samples. $^{32}$P was added 5 min. after addition of virus. In (a), the topmost and lowest curves (O, △) represent RNA and DNA respectively, and the middle curve (●, ▲) LP. In (b), the upper and lower curves (O, △) represent AI and AO respectively, and (●) = virus release. Multiplicity of adsorption = 2-3. The arrow (△) indicates the end of the latent period obtained by extrapolation of a logarithmic plot. (c) Represents a separate experiment performed identically with (a) and (b). The upper curve in (c) is total S-T RNA; the lower is the result of extraction with hot 10% NaCl followed by separation of S-T RNA from the extract (O, △) and the residue (●, ▲).
Table 1. ‘Relative specific activities’ (R), total phosphorus (μg. P) and total \(^{32}\)P (counts per min.=c/m) contents of Schmidt-Thannhauser phosphate fraction during \(^{32}\)P incorporation by chick-cell monolayers maintained in ES/4 % horse serum at 37° in a CO\(_2\)-air mixture.

\(^{32}\)PO\(_4\) was first added to the once-washed monolayers 20 hr. after their formation, and each sample represents one monolayer (2 x 10\(^7\) cells).

| Hours after addition of \(^{32}\)PO\(_4\) | c/m | μg. | P | R | c/m | μg. | P | R | c/m | μg. | P | R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0-1 | 234 | 199 | 1.29 | 342 | 7.4 | 0.049 | 145 | 11 | 0.014 | 0 | 6.6 | 0 |
| 2-66 | — | — | — | 5580 | 7.4 | 0.50 | 935 | 17 | 0.058 | 137 | 7.7 | 0.018 |
| 5-17 | 219 | 173 | 1.32 | 6960 | 7.4 | 0.99 | 2520 | 19 | 0.14 | 433 | 8.6 | 0.053 |
| 7-58 | 206 | 184 | 1.18 | 8000 | 9.0 | 0.94 | 3060 | 15 | 0.21 | 640 | 7.4 | 0.091 |
| 11-42 | — | — | — | 6760 | 5.4 | 1.32 | 3760 | 19 | 0.21 | 1125 | 6.6 | 0.18 |
| 22-66 | 182 | 188 | 1.07 | 7970 | 5.0 | 1.63 | 5930 | 20 | 0.28 | 2080 | 6.3 | 0.35 |
| 27-5 | 202 | 224 | 0.95 | 8220 | 5.2 | 1.66 | 6780 | 27 | 0.26 | 8360 | 6.5 | 0.38 |

In the ‘loss’ experiment of Fig. 3 it can be seen that the gain in mI \(^{32}\)P is slightly greater than the drop of AI\(_R\), which is unexpected as some AI\(_R\) is lost to AO and LMP by synthesis; it is probably due to the small loss from LP, PP and possibly AO noticeable in Table 2 and Fig. 3.

As with Micrococcus pyogenes (Staphylococcus aureus) (Mitchell & Moyle, 1953) AI\(_R\) here is probably a pool supplying most of the cellular P; the R values of the increase of AO and LMP mentioned in the next sections suggests that AI is an intermediary between mI and organic phosphate. The way in which this pool is retained by the cell is discussed separately (Cooper, 1957b).

Source and function of AO. The increment in AO between the mean values for 0-8 hr. in Table 1 (1662 cts./min. and 15.5 μg. P) and 11-28 hr. (5290 cts./min. and 22 μg. P) is 3628 cts./min. and 6.5 μg. P, so that its R value is 0.59; this amount of phosphorus at such a specific activity is clearly only derivable largely from mI or AI, whose specific activity is about unity between these times. Some P may be derived from the relatively unlabelled LMP by lysis or acid-lability. This same figure of 0.59, rather than a value nearer one, and the low specific activities of the initial increments make it likely that most AO derives from AI rather than mI.

In some ‘gain’ experiments the R values of the increments of AO were much higher than 1 (2-8), indicating a high rate of replacement of P by \(^{32}\)P;
while LMP was increasing and was low in ³²P this could be due to AO acting as intermediary between AI and LMP, or by reciprocal exchange between AI and AO. Such an exchange is not suggested by the loss experiment of Fig. 3, where the drop in AO ³²P is not reciprocated by a gain in LM ³²P but by a gain in mAO ³²P. On the other hand, much less AO ³²P was lost to the medium in the experiment of Table 3a. The stability of about half the AO ³²P even after 23 hr. in this experiment (AOi) suggests that not all the AO is available as a metabolic pool.

It is possible that at least some AO is a part of LMP easily separated by TCA rather than, like inorganic phosphate (Cooper, 1957b) free as small molecules in the cell; in this case the particular acid-labile LMP is restricted to the sucrose-soluble fraction (Table 3a).
It is interesting that the bulk of the P accumulates in the AO (Table 1), and is therefore reminiscent of Micrococcus pyogenes in absence of nitrogen source (Mitchell & Moyle, 1953); it is as though the major limitation in the P syntheses of this system occurs at the stage of incorporation into LMP, particularly DNA (see below). However, this appears to be no deterrent to virus growth.

Source of LP and RNA + PP + 7'P. It is difficult to indicate the sources of these fractions because of the presence of components which are able to equilibrate with AI, particularly in the LP and PP fractions (Table 2), and which therefore produce the high R values of increments characteristic of high exchange rates. This is particularly so for the specific activity of the mean increment between the first half (0–7 hr.) and the second half of the experiment of Table 1, and was even more noticeable if either serum or both serum and glucose were omitted, when marked lysis of LMP occurred. On the other hand, the R values of the early increments (when the cells were in better condition) of LP and RNA + PP + 7'P were all low, suggesting that the more stable portions of these fractions (particularly RNA) are derived from AO rather than AI or mI. The evidence, however, is not good for this; it is unsatisfactory to base any estimation of the source of RNA P on the specific activities of heterogeneous groups of substances, such as ‘AO’ and ‘RNA’, rather than the individual nucleotides presumed to be concerned. It is clear from the low activity of DNA that RNA P cannot mainly be derived from DNA P. It is noteworthy that the exchange rate of LP is much lower than that of Micrococcus pyogenes (Mitchell & Moyle, 1953), where LP may be concerned with phosphate uptake mechanisms.

Source of DNA. This cannot be determined from these experiments, since very little DNA was synthesized under the conditions used. It is quite likely that the bulk of the 32P observed in the ‘DNA’ fraction is due to the contaminating residue of RNA left by the somewhat inefficient alkaline hydrolysis (except in Table 2), and the scatter frequently present in ‘DNA’ 32P supports this. It should be noted that the lack of DNA incorporation is probably due to the large number of cells added to each plate (2 × 10^7), as less than this number can be seen to increase in numbers.

Comparison of P metabolism of normal and virus-infected cells

As the R values of increments are too inaccurate to detect small changes, the complementary ‘gain’ and ‘loss’ experiments are compared during one-step growth. Stimulation, blockage or reversal of the numerous P pathways by virus infection might be found by various fractionations.

Schmidt-Thannhauser fractionation of whole cells. The ‘gain’ experiments (Fig. 2) showed that VS infection had no detectable effect during the latent and early release phases on 32P uptake in AI, AO + 7'P, LP, RNA + PP or ‘DNA’ fractions; no effect in the bulk RNA fraction was found when it was extracted with 10% NaCl (Fig. 2c) by the method of Davidson & Smellie (1952). AI equilibration with mI was unaffected during ‘loss’ experiments (Fig. 3, Table 2) (see also Cooper, 1957b) and so was total DNA. A barely
significant increase in AI about the end of the latent period (Fig. 3) may be derived from the LMP and particularly the RNA+LM 7'P fraction (Table 2a). After 23 hr. the $^{32}P$ of the infected cells was nearly all in the medium, mostly as inorganic and acid-soluble organic phosphates. There was a very significant increase in medium LMP (Fig. 8) which coincided with virus release; the AI was not lysed. The use of fully labelled cells (Table 2) takes into account fractions (such as the DNA) which are not fully labelled by incorporation into monolayers as in Fig. 3.

**Fig. 4**

A $^{32}P$ 'gain' experiment followed by cell fractionation by the citric acid method. (•) = virus release. Open circles represent control and triangles infected samples, the upper curve of which shows the citric acid-soluble fraction and the lower the nuclei. The arrow indicates the end of the latent period obtained from extrapolation of a logarithmic plot. Multiplicity of adsorption = 5-6.

**Fig. 5**

A $^{32}P$ 'loss' experiment followed by cell fractionation by the citric acid method. Circles represent control and triangles infected samples. (a) shows virus release (•) and release of $^{32}P$ to the medium. (b) shows the citric-acid soluble fraction (○, △) and the nuclei (●, ▲). The arrow indicates the end of the latent period obtained from extrapolation of a logarithmic plot. Multiplicity of adsorption = 5-6.

**0·1M-Citric acid fractionation.** This method in my hands separated an impure nuclear fraction (cell-free but with attached wisps of cytoplasm) and a citric-acid soluble fraction, comprising the acid-soluble nuclear components (small, Table 3) plus the rest of the cell. 'Gain' experiments employing this fractionation (Fig. 4) showed that total P uptake ceased in both fractions during the exponential release period, while the complementary 'loss' experiment (Fig. 5) showed no big loss from nuclei or cytoplasm as a whole. Increased $^{32}P$ release into the medium during this period was equivocal but was marked after 24 hr., both nuclei and soluble fractions being much depleted in both experiments. Unstained nuclei isolated towards the end of exponential virus release were microscopically identical with controls, the nuclear membrane showing no gross damage.

**Potter-Elvehjem homogenization.** Cells were either homogenized in 0·25 M-sucrose, giving good separation of mitochondria and microsomes but allowing easier rupture of nuclei (Hogeboom et al. 1953); or in 0·25 M-sucrose + 0·0018
Table 2. Effect of vesicular stomatitis virus infection on the rate of $^{32}$P loss (one step 'loss' experiment) from monolayers made from embryos uniformly labelled in ovo.

Multiplicity of adsorption = 4.5 pfu/cell. Table 2 (a) changes in cellular $^{32}$P, (b) changes in medium $^{32}$P. The monolayers had been formed for 20 hr. in non-radioactive medium and washed, so that AI$_n$ $^{32}$P and AO$_n$ $^{32}$P had been removed before addition of virus. C = control monolayers, V = virus-treated monolayers. Figures represent a percentage of the total $^{32}$P of a culture (medium + cells).

<table>
<thead>
<tr>
<th>Hours after inoculation</th>
<th>AI$_1$</th>
<th>AO$_1$</th>
<th>LP</th>
<th>Table 2a</th>
<th>RNA + LM7P</th>
<th>DNA</th>
<th>pfu/cell present in medium</th>
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<td>7.2</td>
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<tr>
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<td>4.0</td>
<td>7.7</td>
<td>9.0</td>
<td>23.7</td>
<td>22.5</td>
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<td>20.9</td>
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<td>19.9</td>
</tr>
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<td>4.2</td>
<td>8.4</td>
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<td>18.6</td>
<td>21.1</td>
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<tr>
<td>5.0</td>
<td>4.9</td>
<td>4.5</td>
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<td>6.6</td>
<td>20.3</td>
<td>16.0</td>
<td>17.7</td>
</tr>
<tr>
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<td>2.5</td>
<td>2.1</td>
<td>8.1</td>
<td>8.4</td>
<td>19.3</td>
<td>18.6</td>
<td>17.7</td>
</tr>
<tr>
<td>7.5</td>
<td>5.1</td>
<td>5.0</td>
<td>5.8</td>
<td>7.3</td>
<td>16.8</td>
<td>17.2</td>
<td>12.7</td>
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</table>

<table>
<thead>
<tr>
<th>Hours after inoculation</th>
<th>AI$_m$</th>
<th>AO$_m$ + 7P</th>
<th>LP</th>
<th>Table 2b</th>
<th>PP + RNA + LM7P</th>
<th>DNA</th>
<th>pfu/cell present in medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>V</td>
<td>C</td>
<td>V</td>
<td>C</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2.6</td>
<td>0</td>
<td>4</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3.8</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>2</td>
<td>7</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>9</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>14</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>
m-CaCl₂, in which nuclei are less fragile but mitochondria and microsomes are not separable. The gain experiment in Ca-free sucrose (Fig. 6) showed that ³²P uptake by microsomes and soluble fraction was halted at the beginning of or during exponential virus release, although mitochondria were not affected. In this experiment the whole cells present in the nuclear fraction masked any changes there. The corresponding ‘loss’ experiment (Fig. 7) showed no

change in nuclei, mitochondria or microsomes, but there was a small drop in the sucrose-soluble fraction and a small increase in the medium during virus release, both barely significant. The 2–8% more of total ³²P regularly found in the medium of infected cells (mostly as inorganic P) would be apparent as a loss in the curves for the particulate fractions if it derived from them, but it would be masked if it were from the soluble fraction.

The sucrose-CaCl₂ ‘loss’ experiment is not presented, as no changes occurred in any control or virus fraction (particularly nuclei) up to a release of 135 pfu/cell.

With or without CaCl₂, the isolated nuclei were microscopically unchanged by virus release, and the microsomal and mitochondrial pellets after centrifugation were identical with controls.
Table 3a. Effect of VS infection on the Schmidt-Thannhauser (S-T) \( ^{32}P \) distribution in the fractions obtained by Potter-Elvehjem cell-homogenization in 0.25 m-sucrose

Cells were harvested from 6 control and 6 infected monolayers 7 hr. after the initiation of a one-step 'loss' experiment (multiplicity of adsorption = 6 pfu/cell, titre at harvesting = 3.88 x 10^8 pfu/ml or 97 pfu/cell, a typical yield for 3-times washed monolayers). Figures represent per cent total \( ^{32}P \) of culture (medium + cells) present in that fraction for C = control and V = virus-treated monolayers. \( P = \frac{(V-C)}{C} \times 100 \) with C and V calculated to 3 decimals.

<table>
<thead>
<tr>
<th>S-T Fraction</th>
<th>Nuclei</th>
<th>Mitochondria</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>V</td>
<td>P</td>
</tr>
<tr>
<td>AI</td>
<td>0.34</td>
<td>0.36</td>
<td>+0.76</td>
</tr>
<tr>
<td>AO + 7'P</td>
<td>0.15</td>
<td>0.13</td>
<td>+0.27</td>
</tr>
<tr>
<td>LP</td>
<td>0.91</td>
<td>0.85</td>
<td>-0.73</td>
</tr>
<tr>
<td>RNA</td>
<td>1.24</td>
<td>1.21</td>
<td>-0.24</td>
</tr>
<tr>
<td>PP</td>
<td>0.66</td>
<td>0.58</td>
<td>+0.65</td>
</tr>
<tr>
<td>LM 7'P + PP</td>
<td>0.47</td>
<td>0.47</td>
<td>+0.85</td>
</tr>
<tr>
<td>DNA</td>
<td>0.24</td>
<td>0.24</td>
<td>+0.85</td>
</tr>
<tr>
<td>Total</td>
<td>3.33</td>
<td>3.73</td>
<td>+1.55</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S-T Fraction</th>
<th>Sucrose-soluble</th>
<th>Medium</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>V</td>
<td>P</td>
</tr>
<tr>
<td>AI</td>
<td>15.4</td>
<td>16.8</td>
<td>+1.89</td>
</tr>
<tr>
<td>AO + 7'P</td>
<td>7.20*</td>
<td>7.23*</td>
<td>+0.43*</td>
</tr>
<tr>
<td>LP</td>
<td>1.33</td>
<td>1.65</td>
<td>+0.35</td>
</tr>
<tr>
<td>RNA</td>
<td>0.06</td>
<td>0.08</td>
<td>+0.02</td>
</tr>
<tr>
<td>PP</td>
<td>2.35</td>
<td>2.07</td>
<td>-0.28</td>
</tr>
<tr>
<td>LM 7'P + PP</td>
<td>3.67</td>
<td>2.90</td>
<td>-21.0</td>
</tr>
<tr>
<td>DNA</td>
<td>1.06</td>
<td>0.66</td>
<td>-37.8</td>
</tr>
<tr>
<td>Total</td>
<td>34.8</td>
<td>33.8</td>
<td>-2.83</td>
</tr>
</tbody>
</table>

* Not significantly altered after removal of 7P.

Table 3b. Some relationships between pfu and LM \( ^{32}P \) in the medium of the experiment described in Table 3a, after 10 min. centrifugation at 1000 rev./min.

The LM\(^{32}P\) was separated by precipitation and washings with TCA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total counts/min.</th>
<th>Total pfu in supernatant</th>
<th>Recovery of pfu (%)</th>
<th>Recovery of LM (^{32}P) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) After 10 min. at 1000 rev./min.</td>
<td>2.2 x 10^8</td>
<td>504</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(2) 100 mg. washed infusorial earth added to 5 ml., stirred for 5 min. and centrifuged for 2 min. at 1000 rev./min.</td>
<td>6.41 x 10^6</td>
<td>273</td>
<td>63.2</td>
<td>54.2</td>
</tr>
<tr>
<td>(3) 100 mg. fresh infusorial earth added to supernatant, centrifuged 16,000 rev./min. for 30 min. Supernatant removed for assay</td>
<td>3.23 x 10^4</td>
<td>24.4</td>
<td>3.7</td>
<td>5.6</td>
</tr>
<tr>
<td>(4) Sediment from (3) resuspended in 5 ml. ES/Ox, centrifuged 2 min. at 1000 rev./min. Supernatant removed for assay</td>
<td>1.47 x 10^4</td>
<td>363</td>
<td>17.2</td>
<td>77.7</td>
</tr>
</tbody>
</table>
Phosphate pathways and virus growth

The sucrose-soluble RNA during virus release. The unchanged total particulate $^{32}$P (gain or loss) and the just significant changes in sucrose-soluble and Schmidt-Thannhauser $^{32}$P (loss) made more information necessary, particularly regarding RNA. For this purpose the cells from six control and six infected monolayers of a 'loss' experiment were removed and pooled 7 hr. after infection, fractionated (Potter-Elvehjem) in 0.25M-sucrose, and the centrifuged products further treated according to Schmidt-Thannhauser. Table 3a shows the $^{32}$P content of each fraction. The handling errors are less

Table 4. Distribution of $^{32}$P between sucrose-soluble and insoluble fractions after freezing and thawing intact cells and also particulate fractions of cells previously separated by the Potter-Elvehjem method (0.25M-sucrose)

<table>
<thead>
<tr>
<th>Nature of the fraction to be frozen</th>
<th>Sucrose-soluble</th>
<th>Sucrose-insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells (slow spin)</td>
<td>TCA-soluble</td>
<td>TCA-insoluble</td>
</tr>
<tr>
<td></td>
<td>66-2</td>
<td>15-5</td>
</tr>
<tr>
<td>Whole cells (fast spin)</td>
<td>59-9</td>
<td>40-1</td>
</tr>
<tr>
<td>Nuclei (slow spin)</td>
<td>1-05</td>
<td>0-17</td>
</tr>
<tr>
<td>Mitochondria (slow spin)</td>
<td>6-27</td>
<td>11-24</td>
</tr>
<tr>
<td>+ microsomes (fast spin)</td>
<td>2-62</td>
<td>1-68</td>
</tr>
<tr>
<td>Sucrose soluble (slow spin)</td>
<td>52-0</td>
<td>6-46</td>
</tr>
</tbody>
</table>

than might be expected, as the recoveries of total $^{32}$P differed between control and infected cells only by 0.34%. The standard deviations of the ratio of difference between infected and control to control of all the experimental values (excluding 'DNA', which is probably RNA contamination and liable to scatter, and medium LMP, in which the infected is much higher than control) was 17-8 and the mean was -4-8. This value of the mean close to zero may indicate that, except for 'DNA', no big errors exist. Of the infected RNA fractions, which should show largest virus effects, the biggest change was the drop in sucrose-soluble RNA ($p=0.05$). One cannot be sure of such small differences, however. The bulk of the LMP in the medium after low-speed centrifugation sedimented at the same rate as the pfu (Table 3b).

In order to confirm this small drop the sucrose-soluble RNA was followed during gain and loss experiments. Cell rupture by one freeze-thaw in sucrose gave a similar soluble fraction (Table 4) to that from Potter-Elvehjem rupture; some extra material may dissolve from subcellular particles, but freeze-thawing the Potter-Elvehjem soluble fraction made nothing insoluble. The sucrose-soluble fraction was therefore prepared by freezing and thawing the
intact cells in 0.25m sucrose for these experiments. Fig. 8 shows a decrease of 30–50% in the sucrose-soluble RNA in loss and gain experiments, which is probably significant, with the complete halt of 32P uptake during gain also found in most other 'gain' fractions. No early stimulation of uptake occurred.

Fig. 8. A 32P 'gain' (a) and a 32P 'loss' (b) experiment performed on the same batch of plates, followed by preparation of the sucrose-soluble fraction by freezing and thawing in 0.25m sucrose and centrifuging for 1 hr. at 25,000 r.p.m. The supernatant was separated by the Schmidt-Thannhauser procedure to obtain alkali-labile acid-insoluble phosphate which was then freed from phospho-protein phosphate and LM 7P. The remainder was assayed as sucrose-soluble RNA 32P (C, △), where circles represent control and triangles infected samples. (●) = virus release. The arrows indicate the ends of the latent periods obtained from extrapolations of logarithmic plots. Multiplicity of adsorption = 5–6.
DISCUSSION

The normal phosphate pathways, indicated in outline above, are complex, as may be expected. In general, phosphate entered the cell as inorganic phosphate via the inorganic pool which was in equilibrium with the external medium, and seemed likely to enter RNA in particular via $\text{AO}_{2}$ rather than direct from $\text{AI}_{5}$. However, both organic fractions were heterogeneous, and it is not correct to deduce the RNA source without examining individual nucleotides.

The present data do not indicate which nucleic acid fraction was concerned with virus growth. The lack of DNA synthesis in control and infected cells, while virus increased at least as well as in growing cells, may be a useful simplification in that some pathways were but little used; otherwise it indicates that any viral DNA synthesis (equivalent to less than 0.5% of the DNA of the cells), or conversion of cellular DNA to viral material which was subsequently released, was too small to be detected. The several RNA's appear similar in that no marked stimulation of incorporation was noticeable either in bulk RNA, sucrose-soluble RNA or in the total $^{32}\text{P}$ of the centrifugal fractions, and no loss occurred in any particulate RNA fraction after virus release. The intact appearance of the isolated cell nuclei after virus release indicates that, if growth occurred intranuclearly, then release from the nucleus cannot occur as a 'burst'. The same applies from the chemical data to the intact cell and to all cell particulates.

Nevertheless, certain changes did occur, the significance of which is not immediately apparent. There was a general halt in $\text{P}$ uptake into all fractions (save possibly the mitochondria) soon after 1 pfu was released per infected cell. The small somewhat equivocal decrease in sucrose-soluble RNA $\text{P}$ was nevertheless apparent in all experiments designed to show it, and probably represents a lysis as there was a corresponding but barely significant increase in small molecular phosphate. Its appearance as RNA in any other fraction, including the medium, would have been apparent. The significance of this for virus growth may be slight, however, as it is pointed out (Cooper, 1957a) that, due to the effect of multiplicity of infection on latent period, a small proportion of the cell population will be at a considerably more advanced stage of viral development than the average. This small lysis may therefore represent the first stage of degeneration in this minority. A probably more significant finding is the presence of RNA $^{32}\text{P}$ in the medium which is adsorbed to infusorial earth and centrifuged at the same rate as the pfu. Some rearrangements of the cellular ribonucleotide ratios have been found during VS infection of chick cells (Dr G. L. Turco, personal communication).

Clearly more data is required to interpret these findings. The fact that the total P content of the matured infective virus must be small compared with the whole cell need not necessarily lead us to expect small changes in the cell. It is necessary to know among other things whether the latent period involves quiescent organization or vigorous viral growth, whether the virus particle contains DNA or RNA and how much, what is the ratio of active to
inactive particles in the final yield, and which fraction is entered by invading virus nucleic acid. Such information would then guide the more detailed chemical work required to disentangle the small changes incurred by virus action in this system.

It is a question for further work as to whether a cell-virus system showing bigger changes is necessarily a better model for examination, since such changes may be non-specific, and may serve only to obscure changes relevant to virus synthesis.

I am very grateful to my colleagues at the California Institute for stimulating discussions, in particular to Dr R. Dulbecco and Dr G. W. Beadle, and to the Gosney Fund Board of Trustees, the American Cancer Society Inc., and the American Cancer Society, California Division, for financial support as a Research Fellow. I am indebted to the Wright-Fleming Institute of Microbiology, St Mary's Hospital, London, for leave-of-absence.

REFERENCES


(Received 28 February 1957)
An Osmotic Barrier for Inorganic Phosphate in Chick Embryo Cells and its Stability during the Latent and Release Periods of Infection by Vesicular Stomatitis Virus

By P. D. COOPER

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SUMMARY: An osmotic barrier for phosphate very near the visible surface of the chick-embryo cell appears to regulate the reciprocal exchange of inorganic orthophosphate between the medium and a component of the acid-soluble inorganic phosphate of cells kept in monolayer culture. Some non-reciprocal transfer of inorganic phosphate occurs, which may or may not be due to cell damage, and the apparent phosphate-impermeable volume decreases by half after 3 hr. contact with phosphate at 0°. At 2° the exchange is at least 97% inhibited. The phosphate-impermeable volume after cell rupture is less than 10% of the intact-cell value, when internal phosphate is released in a form shown by mild separation to be mostly inorganic orthophosphate. Cells lose internal phosphate more slowly in absence of external phosphate, and the addition of external phosphate increases the loss rate to a maximum at physiological concentrations and higher.

Such a barrier provides information on the nature of the cell surface, and shows that adsorption, penetration and release of vesicular stomatitis virus occurs without any detectable damage to the surface controlling the phosphate-exchange rate.

Workers generally agree (e.g. Kamen & Spiegelman, 1948; Sacks, 1948; Mitchell & Moyle, 1953) that concentration of inorganic phosphate into cells does not depend much on simple diffusion alone, but also involves enzymic mechanisms which are possibly phosphorylative. The major uncertainties reside in whether (a) these are exclusively surface mechanisms, so that internal orthophosphate is free within an acid-labile osmotic barrier, or whether (b) the phosphate diffuses freely, to be immobilized enzymically by some intracellular acid-labile matrix. Sacks (1948) believed the evidence indicated possibility (a) for animal cells, but he considered only the alternative of simple physical diffusion (i.e. with no enzymic mechanism) rather than (b). Similar possibilities have been discussed by Mitchell (1953) for Micrococcus pyogenes (Staphylococcus aureus) under the titles of (a) exchange-diffusion and, (b) exchange adsorption, although simple physical adsorption does not apply in the cases discussed below. Exchange diffusion was preferred, as the cells appeared completely impermeable to phosphate in the cold, but freely permeable when the osmotic barrier was destroyed by acid or 5% (v/v) butanol in water. Some substances inhibited exchange at concentrations too low for stoichiometric displacement of adsorbed phosphate, but this does not disprove intracellular fixation, as it only indicates that a catalytic rather than a physical adsorption fixation mechanism is operating. More recent evidence is available to indicate

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that phosphate and other ions exist in the free state within an osmotic barrier in bacteria (Mitchell & Moyle, 1956).

Greenberg (1952) felt at that time that evidence for the mechanism of phosphate uptake by animal cells was inconclusive. The data presented below seem more definitely in favour of the existence of an osmotic barrier for orthophosphate close to the chick-embryo cell wall, through which by definition phosphate ions do not pass freely but require a transport mechanism that turns out to be very dependent on temperature. Such a barrier can provide information on superficial aspects of the viral growth cycle such as adsorption, penetration and release of the virus. Evidence is presented here that these processes during infection by vesicular stomatitis (VS) virus do not detectably affect the rate of phosphate exchange, suggesting that the phosphate barrier remains substantially intact until at least 50% of the virus yield has been released.

**METHODS**

*Virus stocks, virus and $^{32}$P assays, media, preparation of chick embryo cells and monolayers, and the one-step growth curves used in the 'loss' experiments were as described by Cooper (1957a, b).*

**Measurement of phosphate-impermeable volume (Table 1).** Ten 20 hr. cell monolayers were washed with warm phosphate buffer saline (PBS) and the cells removed by 10 min. incubation with 0.25% trypsin in Earle's saline, washed once with cold PBS, chilled thoroughly in ice-water and centrifuged at 500 g for 2 min. in a urine-protein centrifuge tube, accurately calibrated to 0.4 ml. by 0.01 ml. intervals and to 1 ml. by 0.1 ml. intervals. The pellet volume was c. 0.4 ml. The supernatant fluid was sucked off, the remaining volume noted, then 0.5 ml. of cold PBS containing about 0.1 μc $^{32}$P (accurately known) and 150 μg. P was added, and the cells thoroughly resuspended by gentle pipetting. A 0.1 ml. sample ('1st sample') was diluted into 1.9 ml. PBS for total $^{32}$P and haemocytometer cell counts; the suspension contained single cells free of debris with a few clumps of 2-3 cells. This sample gives the total number of cells in the preparation, and comparison of its $^{32}$P content with that known to be added gave the efficiency of mixing (complete within experimental error). Since rate of P exchange did not increase above 0.8 mM (Fig. 2), the use of the higher phosphate concentration of PBS (10 mM) rather than Earle's saline minimized loss of $^{32}$P from the supernatant fluid by reciprocal exchange. Any loss was then due to diffusion into the cell; keeping the cells at 0° (see below) decreased any exchange still further. After sampling ('1st sample'), the tube was centrifuged for 2 min. at 500 g in an ice-filled cup. A 0.1 ml. sample ('2nd sample') was removed from the supernatant fluid for $^{32}$P assay and the pellet volume noted. The cells were resuspended and the whole contents frozen in dry-ice + ethanol mixture and thawed (one cycle), when the cell debris was centrifuged at 1000 g for 5 min. and the supernatant was assayed for $^{32}$P ('3rd sample'). Finally the sediment was resuspended and a sample was taken for intact cell count ('4th sample'). The experiment was performed with a short (3 min.) and a long (180 min.) interval between addition of $^{32}$P and centrifuging.
Virus growth and the osmotic barrier

RESULTS

Temperature dependence of phosphate exchange

A $^{32}P$ 'loss' experiment (Cooper, 1957) was performed, one set of washed radioactive monolayers overlaid with non-radioactive fluid medium being held at 37° in a CO$_2$ incubator, and another identical set at 3° in a vacuum desiccator filled with a 5 % (v/v) CO$_2$ in air mixture. The rate of $^{32}P$ release in the cold plates during the first 4 hr. was about 3 % of the rate at 37°, indicating that simple diffusion did not play much part in the release and that phosphate exchange at 0° was likely to be negligible. This was to be expected from the suspected enzymic nature of the exchange, and did not exclude 'exchange adsorption', but its demonstration was relevant for the experiment to be described next.

Phosphate-impermeable volume

Determination of this volume depends upon the changes in extracellular $^{32}P$ concentration observed when a known amount of $^{32}P$ in a known volume is added to a cold thick cell suspension, using the procedure described in Methods. The results are given in Table 1, and indicate that under these conditions the phosphate-impermeable volume of $18.4 \times 10^7$ cells was 0.19 ml. (3 min. contact), so that the average phosphate-impermeable volume/cell was $1080\, \mu^3$ (radius = 6.26 $\mu$). These are very similar values to those of Dulbecco & Vogt (1954) for the visible dimensions of these cells (mean = $1180\, \mu^3$, radius = 6.55 $\mu$). The phosphate-impermeable volume is likely to be somewhat less than the total volume of the cells (compare Micrococcus pyogenes (Staphylococcus aureus), Mitchell, 1953), but it is not legitimate to calculate the depth of the barrier below the visible surface from these figures. The intercellular space is within the expected range for non-uniform non-deformable spherical objects.

<table>
<thead>
<tr>
<th>Time</th>
<th>Total $^{32}P$ content of prepn. (cts./min.)</th>
<th>$^{32}P$ content of supernatant (cts./min./ml.)</th>
<th>Therefore total vol. extracellular fluid (ml.)</th>
<th>Therefore phosphate-impermeable vol. (ml.)</th>
<th>Vol. pellet (ml.)</th>
<th>Therefore intercellular space (ml.)</th>
<th>Total No. intact cells (visible count) x $10^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min. contact</td>
<td>$3.50 \times 10^4$</td>
<td>$4.80 \times 10^4$</td>
<td>0.73</td>
<td>0.92</td>
<td>0.19</td>
<td>0.39</td>
<td>0.61</td>
</tr>
<tr>
<td>After 1st sample and centrifuging</td>
<td>$3.43 \times 10^4$</td>
<td>$3.78 \times 10^4$</td>
<td>0.60</td>
<td>0.62</td>
<td>0.02</td>
<td>0.38</td>
<td>0.95</td>
</tr>
<tr>
<td>180 min. contact</td>
<td>$8.04 \times 10^4$</td>
<td>$1.07 \times 10^5$</td>
<td>0.75</td>
<td>0.90</td>
<td>0.15</td>
<td>0.39</td>
<td>0.62</td>
</tr>
<tr>
<td>After 1st sample, freeze thaw and centrifuging</td>
<td>$6.97 \times 10^4$</td>
<td>$8.90 \times 10^4$</td>
<td>0.78</td>
<td>0.80</td>
<td>0.02</td>
<td>0.39</td>
<td>0.95</td>
</tr>
</tbody>
</table>
After 180 min. contact at 0° the apparent phosphate-impermeable volume had decreased to 528 μ³/cell, suggesting that phosphate was able to pass the osmotic barrier slowly under these circumstances, a finding supported by the small but significant leakage of ³²P at 3° and the slow release of ³²P in absence of external phosphate at 37°; thus transfer of phosphate may not be strictly by reciprocal exchange, even in the non-growing cell.

Rupture of the cells, by one cycle of freezing and thawing, immediately (within the centrifuging time of 2–3 min.) diminished the phosphate-impermeable volume to <10 % of the intact volume; the pellet volume of the debris was the same as that of the cells, but the supernatant fluid had become viscous and opalescent.

Phosphate-retaining mechanism

One would think it necessary for cell function that a phosphate-excluding barrier should also be able to retain phosphate already in the cell. That this is so is shown by the relatively slow leakage at 37° in the absence of external phosphate (Figs. 1, 2) and the almost complete retention at 5°, while rupture of the cell by freezing-thawing (Table 4, Cooper, 1957b) or by shearing forces...
Virus growth and the osmotic barrier

at 0° (Table 2a, Cooper, 1957b) liberated much inorganic phosphate within 2 min. in sucrose-soluble form. In one experiment a 0.02 ml. sample of the sucrose-soluble fraction of frozen-thawed radioactive cells was placed on a filter-paper strip soaked in 0.05 M-sodium citrate buffer (pH 6.0) in parallel with a 0.01 ml. 0-1 M-Na₂HPO₄ sample. Without drying the paper, the ends were placed in electrode compartments containing the buffer, the centre portion being immersed in CCl₄, while a current of 5 mA was passed at 800 V for 1.5 hr. The strip was then dried and sprayed with the reagent of Bandurski & Axelrod (1951) and irradiated with ultraviolet light to develop the blue-green colour characteristic of inorganic phosphate. The orthophosphates produced blue-green spots 22 cm. from the origin which coincided with a peak containing 85% of the radioactivity of the preparation and 90–100% of the acid-soluble inorganic ³²P; under these conditions the mobility of the fastest monophosphate, uridylic acid, would be 60% of the orthophosphate. There was no 'streaking' of the radioactivity which would indicate decomposition on the paper.

This procedure therefore separated a large proportion of the cellular ³²P as a fraction with the same mobility as inorganic orthophosphate. The mildness of the extraction and separation methods used strongly suggests that it is in this form in the intact cell, and the quantitative identity of the orthophosphate with the 'acid-soluble inorganic phosphate' fraction, where other ³²P fractions are much smaller, suggests that these fractions are in fact identical. It therefore seems likely, although not proven, that the external phosphate is in reciprocal exchange with an internal pool of free phosphate ions.

Effect of virus infection on the rate of ³²P exchange

Previous results (Cooper, 1957b) showed that, unlike bacteriophage (Puck & Lee, 1954) vesicular stomatitis virus infection had no effect on rate of ³²P release, if the commencement of release corresponded to the initiation of infection, so that the effective period examined was the first half of the latent period. Fig. 3 shows the first hr. in more detail, and also that when the replacement of radioactive by non-radioactive medium was delayed so that the most rapid release of ³²P extended over successively later periods, the lack of viral effect on rate of ³²P release could be demonstrated up to the time that exponential virus release was almost complete; a systematic difference in ³²P release rate of 10% should be detectable.

The characteristics of the exchange

Assuming for Micrococcus pyogenes (Staphylococcus aureus) that the cell-wall transport of phosphate was of the exchange-diffusion type, and that the internal and external pools of inorganic phosphate were homogeneous, Mitchell (1958) derived the theoretical relationship ln \[ \frac{1-R_t}{1-R_i} = kt \] for the process of equilibration during gain or loss experiments. \( R_o \) and \( R_t \) were the 'relative specific activities' of either \( I \), the internal phosphate (i.e. cellular acid-soluble inorganic
phosphate) for 'gain' experiments, or E, the external phosphate for 'loss' experiments at the beginning of the experiments (t = 0) and time = t, respectively.

\[ R_t = \frac{^{32}P_t \times K}{P} \]

where \(^{32}P_t\) = total \(^{32}P\) content of E or I at time = t and P = total phosphate of E or I (constant throughout the experiment). K is defined by the relationship (equation A) which exists at complete equilibration:

\[ \frac{^{32}P_o \times K}{P} = R_o. \]  

Equation (A)

---

Fig. 3. Effect of one-step vesicular stomatitis virus infection on the rate of \(^{32}P\) exchange from labelled 20 hr. chick embryo cell monolayers. Different portions of the virus release curves are covered by the most rapid periods of \(^{32}P\) release. The labelled medium was removed, virus diluted in 0-5 ml. of the labelled medium was added so as to give average multiplicities of adsorption (M) of 6 (a) and 2 (b); after 30 min. 4-5 ml. of the labelled medium was replaced. At various times indicated by the commencement of the release curves after the addition of virus (1-2 and 3-0 hr., for M = 2, and 3-3 hr. for M = 6) the medium was removed, the monolayers washed rapidly twice with warm ES + 4% ox serum (ES/Ox) and 5 ml. non-radioactive ES/Ox added. In one experiment (0-1 hr., (b)) virus was added to the washed monolayers in 5 ml. ES/Ox at the same concentration (10⁹/ml.) as the other curves of (b), so that the multiplicity of adsorption within 20 min. should be about the same (c., 2), and samples were removed at close intervals to examine in detail the period of adsorption (0-1 hr.). The broken vertical line represents the rate of \(^{32}P\) release from ruptured cells. (O and ⬤) = control, and (△ and ▲) = virus-treated monolayers; (▽ and ⬤) = virus release curves. The arrows indicate the end of the latent period (release of 1 pfu/cell) calculated from logarithmic plots.

Fig. 4. Relationship between t and inorganic orthophosphate \(^{32}P\) in external environment for 'loss' experiments, or in internal environment for 'gain' experiments, after equilibration was complete (\(^{32}P_o\)), and at time = t (\(^{32}P_t\)), for the exchange of phosphate across the chick cell osmotic barrier at pH 7 and 37°. Circles = control and triangles = virus-infected monolayers; open points = \(^{32}P\) moving into the cells (gain), filled-in points = \(^{32}P\) moving out of the cells (loss). The data is that of figs. 2 and 3 (Cooper, 1957b); the latent period ended at 3-7 hr. Commencement of equilibration coincided with addition of virus for the loss experiment, but was 0-15 hr. after virus for the gain experiment.
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$R_\sigma$ is defined as $a = 1$, and $^{32}\text{P}_o$ as $= \text{amount of } ^{32}\text{P} \text{ present in } E \text{ or } I \text{ in loss or gain experiments when equilibration was complete. Thus the } '\text{relative specific activities}' \text{ indicate the completeness of equilibration. Mitchell's equation depended on the demonstration that there was negligible net change of } \text{P} \text{ during the period of exchange, as was verified experimentally.}

The data of Cooper (1957b) indicate that synthesis of organic phosphate in the chick-cell culture system was slow compared with the exchange, and that there was little net change in $\text{P}$ during gain or loss experiments. Using the same arguments and definitions, Mitchell's equation can be adapted to the data available from either the loss or gain experiments with chick embryo cells as follows:

At $t=0, \quad R_\sigma = 0$, therefore

$$
\ln \left[ \frac{1 - R_\sigma}{1 - R_t} \right] = \ln \left[ \frac{1}{1 - \frac{^{32}\text{P}_o}{K}} \right] = \ln \left[ \frac{P/K}{P/K - ^{32}\text{P}_t} \right] = kt.
$$

Substituting $^{32}\text{P}_o/K$ for $P/K$ (from equation A):

$$
\ln \left[ \frac{^{32}\text{P}_o}{^{32}\text{P}_o - ^{32}\text{P}_t} \right] = kt.
$$

Equation (B)

A plot of $\log_{10} \left[ \frac{^{32}\text{P}_o}{^{32}\text{P}_o - ^{32}\text{P}_t} \right]$ against time for both the gain and loss experiments presented in figs. 2 and 3 of Cooper (1957b) (Fig. 4), and also for the gain experiments in Fig. 3 above, showed an approximately linear relationship, suggesting a similar mechanism in chick cells to that operating in Micrococcus pyogenes. No regular difference was noticeable between infected and control points.

DISCUSSION

It is felt that no mechanisms other than a phosphate-restrictive cell wall can explain the existence of an apparent phosphate-impermeable volume nearly coincident with that of the cell volume, as the specific volume of the cell constituents (as measured by the phosphate-impermeable volume after rupture) is less than 10% of the cell volume. The very rapid breakdown of this restriction on cell-rupture, together with the close correlation between radioactivity and inorganic orthophosphate after very mild separation, indicates that either the phosphate linkages with any enzymically formed intracellular matrix are unusually labile or that the cell holds its reservoir of orthophosphate in the form of free ions retained by a restrictive cell wall.

It is interesting to discuss the nature of the restriction to phosphate, that is, of the mechanism by which phosphate is allowed to pass the cell wall. It has been pointed out elsewhere (Cooper, 1957b) that the 'reversible' acid-soluble inorganic phosphate behaves as a pool which exchanges with the inorganic phosphate of the medium in a largely reciprocal fashion; with a total cell volume of $1180 \mu^3$ and $2.5 \times 10^{-7} \mu g \text{ P/cell}$ its concentration is at least $6.8 \text{ mm}$ or 7 times that of the medium. It has been possible to set up two hypothetical models to explain the way in which this phosphate is concentrated and
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retained. One was that the cell wall is impermeable to phosphate in the physical sense, retaining as in a bag free phosphate ions whose entry requires a specific transport mechanism, and the other that phosphate diffuses physically into the cell at least as rapidly as the exchange process and is then either immobilized in a phosphate-accepting framework by a chemical process, probably enzymic, or retained by Donnan-type equilibria. These two hypotheses were compared above with 'exchange-diffusion' and 'exchange-adsorption' as defined by Mitchell (1958) for *Micrococcus pyogenes* (*Staphylococcus aureus*).

The fact that the cell appears impermeable to phosphate after only 3 min. of contact with phosphate merely means that any inward physical diffusion is too slow to be measurable within this time. An exponential process 50% complete in 70 min. would be only 3% complete in 3 min. If one can therefore demonstrate, under conditions where any enzymic step is negligible, that the rate of diffusion into or out of the cell at 37° is slower than the exchange rate at 37°, exchange adsorption as defined above is excluded.

It is shown above that liberation of phosphate from the cell is negligible at 0°, and it is mentioned in an appendix that physical diffusion inwards may not predominate at 37°, although more data is required to be certain of this point. These points would therefore favour exchange diffusion.

It is shown above that the kinetics of the exchange are very similar to those of *Micrococcus pyogenes* (*Staphylococcus aureus*), which, can be justified theoretically from consideration of the exchange-diffusion mechanism. It is more than likely that the proportion of the 'exchange' apparently due to physical diffusion is a result of some damage to the cell surfaces during handling. It is interesting that the rate of exchange of phosphate is maximal at about physiological concentrations (Fig. 2); the 50% equilibration time is 70–90 min. Addition of external phosphate to P-free ES at once initiated the exchange (Fig. 1), and subsequent removal allowed only the slow release again.

Having shown that the relatively slow exchange of phosphate with the medium (compared with the much more rapid release or entry after cell rupture) is due to a restriction at the cell surface. Whatever the nature of this restriction, the lack of effect of VS virus infection on this exchange makes it possible to say that both penetration and release of this virus can result in only a very limited lysis of the cell wall. At all events, release is certainly not accompanied by a 'burst' in the sense in which the term is applied to the release of bacteriophage. This conclusion has frequently been drawn from other types of observations with other viruses (Dulbecco, 1955). Similarly, the fact that 32P release is not retarded by the virus shows that any enzymically-dependent transport is not grossly damaged by passage of the virus.

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REFERENCES


APPENDIX

An approximation to the kinetics of the entry of phosphate into the cell at 0° can be obtained from the 'phosphate-impermeable volume' data after 3 hr. at 0° (Table 1). If the same principles apply to a process of reversible diffusion at 0° as to the exchange at 37°, the relationship

\[
\ln \left( \frac{32P_{\infty}}{32P_{\infty} - 32P_t} \right) = k_0 t
\]

will also hold. Presumably the 'phosphate-impermeable volume' does not actually shrink, but rather the data indicates a progressive diffusion of phosphate from the external space (volume = \( V_e \) ml.) to the internal (\( V_i \) ml., = phosphate impermeable volume at \( t = 0 \)). It is further assumed that the cell's internal volume remains constant during the experiment. Then \( 32P_{\infty} \), the \( 32P \) content of the internal space at complete equilibration, = \( T \cdot V_i \), where \( T = \) total \( 32P \) content of the system, since then the internal and external concentrations should be approximately equal (the internal total inorganic phosphate, at c. 7 mM, is of the same order as the external phosphate (10 mM) in this experiment).
In Table 1, the 'phosphate-impermeable volume' is $\frac{1}{2} V_i$ after 3 hr. The external concentration of $^{32}P$ is then $\frac{T}{V_e + \frac{1}{2} V_i}$ and the amount of $^{32}P$ in the external space is $\frac{T}{V_e + \frac{1}{2} V_i}$. Therefore the internal $^{32}P = T - \left[ \frac{T}{V_e + \frac{1}{2} V_i} \right] = \frac{T}{2 V_e + V_i}$. Therefore

$$\frac{^{32}P}{^{32}P_\infty} = \frac{1}{1 - \frac{V_e + V_i}{2 V_e + V_i}} = \frac{2 + V_i}{V_e}.$$  

Therefore

$$\ln \left[ 2 + \frac{V_i}{V_e} \right] = k t \text{ (from equation B).} \quad \text{(Equation C)}$$

where $t' = $ time at which the phosphate impermeable volume is $\frac{1}{2} V_i$. In the exchange experiments of Fig. 4, $V_i/V_e$ is very small and $t' = 1.2$ hr.; the value of $\frac{^{32}P}{^{32}P_\infty}$ is 2 gives a 50% equilibration time of 70–90 min., which in this case therefore coincides with the time at which the phosphate-impermeable volume appears to be $\frac{1}{2} V_i$. From equation (B), $k_0 = 0.58$ hr.$^{-1}$. $V_e/V_i$ is larger in the experiment of Table 1 (c. 0.2), giving, on the basis of the single point at 3 hr., where the phosphate-impermeable volume happens to be $\frac{1}{2} V_i$, a value for $k_0$ for the entry of phosphate at 0° of 0.26 hr.$^{-1}$, or approximately one-half that of the $k$ value for entry at 37° (Equation C). Thus the effect of temperature appears considerably greater than one would expect from a purely physical mode of entry, suggesting that as for 'loss' the physical process is not predominant at 37°.

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SUMMARY: Above a value of one, repeatedly doubling the multiplicity of infection of chick embryo cells by vesicular stomatitis virus progressively shortened the latent period by about 0.6 hr; this phenomenon is referred to as 'shortened latency'. Varying the multiplicity above unity with dilute-passage stocks did not interfere with rate of infective virus release, number of cells infected, or final yield, i.e. there was no 'von Magnus' effect or other obvious interference phenomena.

The doubling time for virus release was also about 0.6 hr. This suggested that virus may have been growing as a simple intracellular pool equally accessible to all adsorbing virus, and that 1 particle was released when the pool reached a certain size (perhaps 20–200 units) irrespective of inocula. However, other explanations are possible, and of those allowing experimental test, earlier initial adsorption of virus, multiplicity reactivation amongst a partly inactivated population, more rapid elution of attached virus or more rapid release of accumulated internal virus could not account for shortened latency.

Earlier results (Cooper, 1957a) suggested that the release of vesicular stomatitis (VS) virus from completely infected chick embryo cell monolayers occurred earlier when inocula of higher titre were used. More specifically, the latent period (defined here as the time between addition of virus and release of one plaque-forming unit or pfu per infected cell) was shorter at higher multiplicities of infection. Final yields and rates of release were not affected.

These findings are confirmed and extended below. For the present purpose, such a phenomenon resulting solely from varying the multiplicity of infection of dilute-passage stocks (other conditions such as temperature and pH being equal) will be referred to as 'shortened latency'. The effect of undiluted-passage stocks will be considered elsewhere (Cooper & Bellett, to be published).

Some quantitative aspects of shortened latency are presented, which have suggested a simple hypothesis for the intracellular increase of viral units; this is elaborated in the discussion of this paper. However, there are several alternative explanations, and an attempt is made to evaluate some of them experimentally.

The possible existence of auto-interference and exclusion, so that not all adsorbed virus may participate in viral reproduction, is very relevant to any explanation of shortened latency. This question is considered separately for VS (Cooper, 1958), and the results suggest that homotypic exclusion does not occur with dilute-passage stocks of this virus. Extensive discussion of exclusion is therefore omitted from the present paper.

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Shortened latency in multiple infection

METHODS

Viral assays and stocks, one-step growth curve methods and media were as described by Cooper (1957a). When infected monolayer cells were removed with trypsin for virus release in suspension or for assay of infective centres, the inoculum was removed, plates washed twice with phosphate buffered saline (PBS, Dulbecco & Vogt, 1954), 2 ml. 0.7 mg. Armour crystalline trypsin/ml. or 2.5 mg. Difco trypsin/ml. added at 37° for 5 min., cells quantitatively resuspended and chilled, washed twice in PBS, and resuspended in 1 ml. of PBS. Large clumps were allowed to settle for 5 min., and the monodisperse supernatant fluid, containing not less than 50% of the total cells, was removed for assay.

Multiplicity of infection relates to the number of pfu adsorbed per cell, and not necessarily to the pfu which actually achieve infection. The multiplicities reached in Figs. 1 and 2 are not dependent upon the plating efficiency of the virus assays (defined as ability to detect infective virus) as the adsorptions for both were carried out in the same manner; an agar cell-suspension method (Cooper, 1955) appeared to detect only 20-40% more infective virus than did the monolayer method. Therefore ‘pfu adsorbed’ is regarded as the same as pfu added in the inoculum.

RESULTS

Effect of multiplicity of infection on latent period

Figures 1 and 2 confirm in more detail the earlier suggestion (Cooper, 1957a) that higher multiplicities of infection gave shorter latent periods with VS virus growing in chick cells. Thus at higher average multiplicities of infection, on the average each infected cell released its first progeny particle sooner. Rate of release and final yield were not affected; it is therefore noteworthy that no interference phenomena (decrease in release rate or final infective yield) were found. This is characteristic of the dilute-passage stocks used; undiluted-passage stocks show marked interference (Cooper & Bellett, to be published). The experiments shown used the New Jersey serotype, but the same phenomenon was also found with the Indiana serotype.

The experiments of Figs. 1 and 2 were performed with chick cell monolayers of the same batch infected simultaneously with different dilutions of the same high-titre virus stock, thus excluding possible day-to-day variations in cells or virus preparations as the explanation of apparent shortened latency. Shortened latency is not due to re-adsorption effects (possible in the monolayer experiment of Fig. 1) as identical results were obtained in dilute cell-suspensions (Fig. 2) where re-adsorption of released virus was negligible. This similarity also suggests that, in general, re-adsorption is not a significant factor in monolayer release curves, which is to be expected from calculations involving adsorption rates (50% of virus is adsorbed in 3 hr. from 5 ml., whereas release is doubled every 30 min.). Figure 3 shows that there was a roughly logarithmic relation between multiplicity and latent period; a possible
significance of this is discussed later. The experiment of Fig. 2 (at 37°) was repeated with the exponential release period at 30° (the latent period having been at 37° as before), when the same shortened latency occurred even though the release doubling time was lengthened to approximately 1 hr.

Possible explanations for 'shortened latency' shown to be unlikely

Virus elution. Virus may elute from the higher multiplicities of adsorption, giving apparently earlier release; in this case the earlier liberation of virus would be the summation of elution and virus release from an effectively low- or single-multiplicity infection. It is necessary to postulate for this either that
the probability of elution of each adsorbed particle is much greater in multiple than in single infections, or the unlikely case that higher multiplicities depress the final yield by a factor exactly equal to the proportion of virus eluting from lower multiplicities, since elution from low multiplicities would reduce the number of infective centres.

![Graph showing the relationship between multiplicity of infection and latent period.](image)

**Fig. 3.** The relationship between multiplicity of infection and latent period derived from Fig. 1 (○) and Fig. 2 (▲), which each used plates of one batch. Three points (●) are from independent experiments with different batches of plates, and the bar at multiplicity of six represents the extreme range of latent periods observed in Fig. 1 of Cooper (1957a).

That elution is not occurring is shown by: (a) the exponential nature of the release curves yielding virus in significant excess over that added, and (b) when virus growth (in a suspension containing in 1 ml. 10⁴ cells previously infected in monolayers to an average multiplicity of 20) was prevented 40 min. after infection by freeze-thaw disrupting the cells or by the addition of 10 mM NaCN, the 10 hr. virus yield/cell and therefore presumably the elutable virus was less than 0.1% of the yield/untreated cell. In a number of experiments, the pfu liberated after 1 freeze-thaw of cells infected with high multiplicities was equivalent to 1–10% of the number of infected cells, or less than 1% of the virus added (Cooper, 1958); this is of the same order as the intact cells probably present (Cooper, 1957b).

**Earlier adsorption of a single particle.** As the rate of VS virus adsorption is probably independent of virus concentration, on average the time at which each cell receives its final quota of virus should be constant and independent...
of multiplicity. However, a higher virus titre in the inoculum would mean that each cell would be likely to receive its first particle in a shorter time. It can be shown that little more virus is adsorbed by monolayers after 30 min. under the conditions used and 50% is adsorbed in 10–15 min., independently of the concentration; therefore for the longest latent period, i.e. for single infection, half the cells have received their virus by 15 min. and nearly all by 30 min. The inoculum was usually removed and the plates washed at this stage. Thus the higher titres of virus added for the higher multiplicities could not achieve single infection more than 30 min. before the low titres. The latent period could not therefore be shortened in this way by more than 30 min., and there should be little difference in latent periods above multiplicity 2–8; reference to Figs. 1 and 2, where the maximum shortening was 3 hr., and where continuing increase in the multiplicity above 8 still further shortened the latent period, shows that shortened latency is not due to earlier adsorption at higher multiplicities.

Multiplicity reactivation among a partly inactivated virus population. It is very likely from the thermal instability of VS virus (Cooper & Bellett, to be published) that freshly harvested virus preparations contained an excess of virus which was non-infective for this system (i.e. not leading to infective progeny and therefore a plaque); many preparations certainly contained an appreciable proportion, often an excess, of particles ‘storage’-inactivated during sojourn at −20°. It can be imagined that these may yield infective progeny under conditions of multiple infection and thereby affect the latent period in certain circumstances. However, direct examination for multiplicity reactivation in the sense of larger recovery of infective centres at high multiplicities of infection compared with low, revealed none in a population containing a tenfold excess of ‘storage’-inactivated and an unknown excess of thermally inactivated particles (Table 1). Alternatively, the penetration of the cell by several particles, active or inactive, may provide material, perhaps structural protein or enzymes, which can overcome some bottleneck in virus production and lead to earlier maturation. In this case pretreatment of the cells with inactive particles might affect the latent period. Reference to Fig. 1 of Cooper (1958), where the cells were pretreated with a large excess of ‘storage’ and uv-inactivated particles, shows no effect on latent period by this means. Similar experiments (Cooper & Bellett, to be published) with an excess of the interfering component present in undiluted-passage stocks also showed no effect on the latent period in those cells able to release virus, and numerous one-step growth curves at single multiplicity (using various stocks and an input multiplicity of 10⁻² to 0.8) always gave a latent period of 4.0–4.5 hr., showing that the varying multiplicities of thermally inactivated virus thereby achieved can have no marked effect on latent period.

Earlier release of an accumulated pool of mature virus

Experimental manipulation of cells infected with virus have in some hands stimulated virus release; there is evidence that mature poliovirus may accumulate in or on cells (Howes & Melnick, 1957) and be released in a burst (Lwoff,
Table 1. The lack of effect of dilution of a dilute-passage seed containing excess of 'storage' and thermally-inactivated virus on the recovery of cells yielding infective progeny (lack of multiplicity reactivation)

Dilutions of a VS virus stock originally assaying $5 \times 10^5$ pfu/ml, and reduced to $6 \times 10^3$ pfu/ml on storage at $-20^\circ$, were added in 0-5 ml. amounts to each of 7 monolayers containing $2 \times 10^6$ cells; after 45 min. the inoculum was removed, plates washed twice with 5 ml. Earle's saline, cells removed with trypsin, chilled, washed once with PBS, re-suspended in 1 ml. and supernatant removed after 5 min. for assay of infective centres on monolayers.

| Seed | Multiplicity of infective VS (pfu) | Minimum multiplicity of inactivated VS | Assay | Expected from pfu added in seed | Plating efficiency (%) Expected infective centres $\times 100$
|------|-----------------------------------|--------------------------------------|-------|-------------------------------|----------------------------------|
| Dilution | | | Dilution | pfu in | pfu in | Observed | Expected in seed
| Neat | 1-5 | 11 | $5 \times 10^{-2}$ | 17, 10 | — | $2-7 \times 10^4$ | 1-08 $\times 10^7$ | 25-0
| 1/3 | 0-5 | 3-6 | $5 \times 10^{-4}$ | 10, 9 | — | $1-9 \times 10^4$ | 5-6 $\times 10^4$ | 34-0
| 1/10 | 0-15 | 1-1 | $10^{-4}$ | 5, 6 | — | $5-5 \times 10^4$ | 2-5 $\times 10^4$ | 22-7
| 1/50 | 0-05 | 0-36 | $3 \times 10^{-4}$ | 11, 10 | 13, 18 | $2-9 \times 10^4$ | 7-5 $\times 10^4$ | 38-7
| 1/100 | 0-015 | 0-11 | $10^{-2}$ | 8, 16 | 20, 22 | $1-1 \times 10^4$ | 2-5 $\times 10^4$ | 44-0
| 1/900 | 0-005 | 0-036 | $3 \times 10^{-3}$ | 8, 13 | 13, 14 | $2-7 \times 10^4$ | 7-5 $\times 10^4$ | 36-0
| 1/1000 | 0-0015 | 0-011 | $10^{-2}$ | 6, 5 | 15, 16 | $7-0 \times 10^4$ | 2-5 $\times 10^4$ | 25-0
High multiplicities of infection with VS virus may possibly have so damaged the cell surface that an accumulated pool of infective virus was released sooner than with low multiplicities. However, this would mean that the non-released infective virus associated with singly infected cells must be at least as numerous as the virus released by the higher multiplicity. Thus non-released virus during single infection would have to exceed the released virus by a factor of 10 or more. In fact, precisely the opposite was found by Franklin (1958) for VS virus growing in chick and monkey-kidney cells, i.e. the released virus exceeded the non-released by a factor of 10, as was found for western equine encephalomyelitis virus by Rubin, Hotchin & Baluda (1955). This implies that any virus particle must be released very rapidly once it is mature (becomes infective).

Further evidence against this idea is that in the experiment of Fig. 2 at least 90% of cells were ruptured before assay by freezing and thawing, so that any non-released pool would be artificially released and included in the total count; it can be seen that the total virus produced by the cells (released plus non-released) still shows shortened latency.

**DISCUSSION**

Some data of Doermann (1952) suggested that intracellular coli-phage T4 appeared slightly earlier with higher multiplicities, although the absence of marked shortened latency in bacteriophage may be explained by the presence of mutual exclusion. Dulbecco & Vogt (1954) found that the latent period of western equine encephalomyelitis virus was shorter at a higher multiplicity. Liu & Henle (1951) found a similar phenomenon with influenza virus (strain LEE), although this might be accounted for by an enzymic release of attached virus in the same way that multiplicity reactivation was simulated (Henle & Liu, 1951), but later shown probably not to occur in this sense (Cairns, 1955). Kaplan (1957) mentioned an earlier release of herpes simplex virus at higher multiplicities, but as, due to technical difficulties, he appeared in some doubt as to the precise number of infected cells present in these experiments one cannot say whether the latent period, as defined in the present paper, was shortened. Darnell (1958) presented data which suggest a longer latent period in single than in high multiplicity for intracellular poliovirus. However, the reverse applied among the multiple infections (i.e. the latent periods were longer for higher multiplicities) and separate infective centre assays (particularly needed for the single multiplicities) were not given, so that shortened latency was not demonstrated. Dr R. M. Franklin (personal communication) found that L cells, infected with fowl-plague virus and subsequently 'stained' with fluorescent antibody, fluoresced earlier the higher the multiplicity.

The results presented above confirm the existence of the phenomenon of shortened latency in VS virus-infected chick cells. Certain possible explanations of the phenomenon seem to be excluded, namely, elution of virus at the higher multiplicity, earlier infection of the culture with seeds of higher titres, multiplicity reactivation of inactivated virus or pooling of certain...
Shortened latency in multiple infection

components to produce a complete particle sooner, and an earlier release of an
accumulated pool of mature virus at the higher multiplicities. These will not
be considered further.

It is demonstrated elsewhere (Cooper, 1958) that homotypic exclusion
probably does not occur with dilute-passage VS stocks, although undiluted-
passage VS stocks can contain a possibly 'incomplete' form which can exclude
homotypic infective VS (Cooper & Bellett, to be published). However, all
stocks used had only a limited number of passages (less than three, all with
dilute inocula in tissue culture) from a picked single plaque, had a high titre
(1-2 x 10^9 pfu/ml.) and showed no depression of yield at higher multiplicities
of infection, and so it is believed that they contain negligible amounts of the
excluding component. There seems therefore no direct evidence for believing
that all adsorbing particles are not participating, at least to some extent, in
the growth cycle; until information on homotypic exclusion can be obtained,
for example, from genetically labelled strains little further can be said on this
subject.

It is proposed to consider briefly some explanations of shortened latency
which assume non-exclusion and which did not allow direct experimental test.
The earlier intracellular appearance of the first infective virus particle in
multiple infection suggests that entry of several particles: (a) is equivalent to
one particle reproducing for longer, (b) enables one particle to start reproducing
sooner, or (c) permits one particle to reproduce faster. Possibilities (b) and (c)
need not imply mutual exclusion between particles entering a cell, and all three
possibilities are not mutually exclusive; (a) and (b) may well occur together.

If (a) is true, for example by multiple contributions to a vegetative pool
(suggested by recombination among certain other animal viruses), then the
following hypothesis is possible.

Let M = number of contributions to the pool (multiplicity of infection) and
assume that increase of viral multiplying units is exponential.

Then r = the number of multiplying units present at the end of the latent
period = M 2^k, where k = (L - d)/g, and L = length of latent period, d = 'dead-
time', during which the virus particle adsorbs, penetrates and organizes into
a replicating state, g = doubling time of pool.

Therefore log M = -L/g log 2 + d/g log 2 + log r.

A plot of log_{10} M against latent period (Fig. 3) gave a straight line of
negative slope, indicating that if d is constant during either of the two series
of growth curves of Fig. 3 then r is constant for the multiplicities studied.
The value of r cannot be determined as d is unknown but the extrapolation to
zero latent period indicates that r is less than 200. The ending of some latent
periods by 1-1.5 hr. means that multiplication has at least started in these
cells by this time, and if d is constant for all multiplications then d is less than
1.5 hr. and r is greater than 20. Calculations of g from both slopes in Fig. 3
gave values of about 0.6 hr., similar values to those calculated for the release
doubling time from the virus release curves (0.54 and 0.60 hr. respectively;
most other experiments gave 0.6 hr.).

In other words, doubling the multiplicity should put the pool one generation
ahead. Therefore in the simple case where final yield and rate of multiplication are unaffected, where the content of multiplying units is constant (20–200) when an average of one particle is released per cell, where vegetative growth and release have the same exponential rate, and where the pool is equally accessible to all adsorbing particles, then doubling the input should decrease the latent period by one release-doubling time. These assumptions are all implicit in this hypothesis, which then suggest a rather low probability of maturation of vegetative units compared with the high probability of release of mature virus found by Franklin (1958).

Possibility (b) may be caused by co-operation between particles in penetration or in organizing a replicating site, or in merely giving a greater probability of success in penetrating or in intracellular collision with a suitable site. Cairns (1957) suggested that in influenza virus infections of the allantoic membrane there is a probability of delay after adsorption in starting an infection at single multiplicity, so that each of a series of such infections will start multiplying at very different times. It is likely that a similar probability of delay occurs among particles multiply infecting a cell, so that the higher the multiplicity the sooner virus growth might be started by one of the adsorbing particles. Possibility (c) is allowed because exponential release may be the result of a series of 'bursts' randomly arranged among a large population of cells, and the actual duplication rate need bear no relation to release. It was mentioned above that an accumulation of mature virus was not found, making the 'burst' hypothesis for release alone unlikely, but several ways can be envisaged in which wholesale maturation and release could occur very rapidly in individual cells.

These questions are fundamental to our concept of viral growth. Means to investigate some of the alternatives discussed above could be devised, but some more direct method is very desirable.

I am greatly indebted to Dr R. Dulbecco and my colleagues at the California Institute of Technology for helpful discussions. I wish to acknowledge financial support for part of this work as a Research Fellow of the American Cancer Society Inc., and the American Cancer Society, California Division.

REFERENCES

Shortened latency in multiple infection


(Received 9 April 1958)
Homotypic Non-exclusion by Vesicular Stomatitis
Virus in Chick Cell Culture

By P. D. Cooper
SUMMARY: Placing live or inactivated vesicular stomatitis virus of one serotype on chick cells in tissue culture prevented most of the cells from releasing infective virus of the other serotype when super-infected with it (heterotypic exclusion). Inactivated virus did not prevent super-infection with the same serotype and had no effect on the latent period or rate of virus release (homotypic non-exclusion and non-interference). The ‘Indiana’ serotype was more effective as heterotypic excluding agent than was the ‘New Jersey’ serotype, and exclusion was noticeable when only 12 min. elapsed between interfering and challenge virus. Each cell liberated virus of only one serotype when infected with live virus of both serotypes, but the serotype released was often (20–40%) not that of the particle first adsorbed. Heterotypic exclusion in fact behaved as if it were reversible and dependent on the multiplicities of infection, at least within the latent period. Many inactivated particles per cell were adsorbed before heterotypic exclusion was achieved.

The phenomenon of ‘shortened latency’ in vesicular stomatitis (VS) virus infections of chick embryo cells (Cooper, 1958), where a higher multiplicity of infection caused the first infective progeny to appear sooner, raised the question of whether one particle of this virus was able to exclude an identical particle from infecting the same cell. Clearly any interpretation of shortened latency is affected by exclusion, since then all adsorbing virus would not be contributing equally to the progeny.

This paper demonstrates the presence of heterotypic exclusion by live or inactivated preparations of VS virus (Indiana or New Jersey serotypes) under conditions where the same inactivated preparations do not exclude the live homotypic virus. The conclusion is drawn that early exclusion between live homotypic particles is therefore also unlikely, although not disproven.

Some interpretations of shortened latency are discussed elsewhere (Cooper, 1958); because of the particular relevance to shortened latency the present paper examines interference mostly in the early latent period.

METHODS

* Present address: Virus Culture Laboratory, Medical Research Council Laboratories, Carshalton, Surrey.
layers as described in the latter paper. The guinea-pig hyper-immune antisera used in separating the serotypes (kindly given by the Research Institute, Pirbright) had no effect on plaque counts of their respective serotypes when incorporated to a concentration of 1/6000 (v/v) in the agar overlay, but completely prevented plaque formation at 1/600; a concentration of 1/120 was routinely used, and its effectiveness usually checked for each experiment. Plaque counts with heterotypic antisera of either type were completely unaffected at 1/60 (v/v).

Ultraviolet inactivation was accomplished by irradiating 1 or 2 ml. of tissue culture fluid for 1–5 min. in a 10 cm. Petri dish at 18° on an automatic rocking device which tilted the dish once every 2 sec. Under these circumstances one lethal hit occurred about once every 40 sec. The dose for distilled water was about 5 ergs mm.−² sec.−¹.

Technique for establishing conditions of exclusion

All experiments were made by adding a primary inoculum to 20 hr. chick embryo monolayers (usually the interfering agent) followed after a period of adsorption by removal of the inoculum, addition of the secondary inoculum (usually the challenge virus) and its removal after a second period of adsorption. These are described where appropriate. Usually cells were washed and removed by trypsinization for assay of infective centres as described below; exclusion is therefore defined and used in the bacteriophage rather than the sperm-ovum sense, i.e. a prevention of a potentially adsorbing and infecting virus from liberating infective progeny characteristic of its own serotype, and therefore from forming a plaque neutralized by its own antiserum.

All operations up to cell chilling were made in an incubator hood, which is a Perspex box with sloping front and sleeved arm-holes designed for manipulating Petri dish monolayers in an atmosphere of controlled temperature, CO₂ content and humidity. Thus temperature, pH and tonicity variations in the monolayers were eliminated during the early latent period, which was both the time under investigation and the time potentially affected by the manipulations. The similarity of one-step growth curves produced in the incubator hood with those produced without it showed that such variations are not important for growth curves provided that they are soon corrected (usually within 15 min.); their elimination was important for the present purpose as otherwise they might be incriminated as potential causes for the lack of homotypic exclusion described below.

Harvesting cells for assay. After the final adsorption of seed, fluid was aspirated and drained as completely as possible, plates were washed twice with PBS (phosphate buffered saline, Dulbecco & Vogt, 1954), and cells removed by 5 min. treatment with 5 ml. trypsin (2-5 mg./ml. in PBS) at 37°. The suspension was thoroughly chilled in ice, centrifuged, washed once in PBS and suspended in 1 ml. cold 50% (v/v) PBS in ‘conditioned’ medium (centrifuged medium from formed 20 hr. monolayers). After 5 min. standing to settle large clumps, the supernatant 0.75 ml. was removed for assay of infective centres and total cells (haemocytometer count). The suspension was
then almost entirely monodisperse. Cells were plated while still in the early latent period; after all plating of cells was complete, free virus was estimated as the infective centres remaining after 3 min. centrifuging at 1000 rev./min. Some estimation of the higher limit of 'associated' virus, i.e. externally, attached plus internally contained mature virus, was obtained as the number of infective centres remaining after one cycle of freezing at —70° and thawing; the relevance of this control is that the externally attached virus may elute and give spuriously high infective centre counts. Such elutable and free virus was always low compared with the total infected cell count. It is felt that the bulk of the 'associated' virus in the present work is accounted for by the undisrupted cells still present (up to 10% of the total).

RESULTS

Heterotypic but not homotypic exclusion by inactivated VS virus

Table 1 shows that when monolayers of chick cells from the same batch were allowed to adsorb a high multiplicity of 'storage'—(—20°) plus ultraviolet-inactivated (UVI) virus particles of either New Jersey (NJ) or Indiana (IND) serotype, 100% of the cells thus infected could produce progeny from a single homologous active particle added subsequently, but only 20% (IND VS as primary inoculum) or 40% (NJ VS as primary inoculum) from a single subsequent heterologous particle. This lower efficiency of NJ VS as an interfering agent was evident in all of three experiments where it might be found, despite a frequently higher multiplicity of infection by the NJ VS.

In these experiments the interfering agent was given the greatest opportunity of establishing itself, consistent with limitation to the early part of the latent period, namely, adsorption for 1 hr., frequent respreading of the inoculum, and high multiplicity of infection. It can be seen that, despite this maximum opportunity, the heterotypic exclusion is not efficient in the sense that by no means all the cells having the interfering agent are prevented from producing progeny. The primary inoculation was carried out by a method which had been shown with live virus to infect nearly all of the cells (Cooper, 1955, 1957), so that all cells are likely to receive inactivated virus. Nevertheless, although heterotypic exclusion is not complete, its presence in 60–80% of the cells rules out the possibility that the non-appearance of homologous exclusion in the same experiment was due to some failure of the majority of cells to be infected by the primary inoculum, for example, that the inactivated virus did not adsorb.

Since these preparations are certain to contain much heat-inactivated virus (the half-life of VS at 37° is 1–3 hr. in the usual growth media, Cooper & Bellett, to be published), the lack of homotypic exclusion applies to heat-inactivated as well as to UVI and storage-inactivated virus.

The heterotypic exclusion by inactivated NJ or IND strains was reciprocal, but whether 'mutual exclusion' occurs in the sense that only one but not both serotypes can grow together in the same cell will be discussed below. As was found repeatedly in other experiments, no multiplicity reactivation was
Table 1. Effect of pretreatment with homotypic inactivated VS virus on infected cell count

As with all tables in this paper, the data relate to one experiment with one batch of plates and virus seeds. The seeds used here initially contained 10⁶ pfu/ml. (NJ) and 2·0 x 10⁶ pfu/ml. (IND) and had inactivated to 2·8 x 10⁶ pfu/ml and 6·1 x 10⁶ pfu/ml respectively at -20⁰; portions of these stocks were then ultra-violet irradiated to 10⁻⁸ (NJ stock, 9 lethal hits) and 10⁻⁴ (IND stock, 12 lethal hits) survivors. Primary inocula (0·5 ml.) were respread 3 times during 1 hr. at 37⁰; after removal of this but without washing, secondary inocula (0·5 ml.) were added for a further 30 min. when the cells were washed and resuspended to 1 ml. for assay. M = multiplicity of infection with live, or 'stor' = storage and 'UVI' = ultra-violet inactivated virus (the fraction indicates ratio of added infective virus particles to total cells in the monolayer). Plaque assays at 10⁻³ of monolayer 6 with IND antiserum in the agar overlay gave 2 plaques, and of monolayer 8 with NJ antiserum gave no plaques.

<table>
<thead>
<tr>
<th>Monolayer no.</th>
<th>Primary inocula (inactivated)</th>
<th>Secondary inocula (live)</th>
<th>Total cells recovered x 10⁷</th>
<th>Cells intact (pfu) at 10⁻⁴ per ml. x 10⁶</th>
<th>After freeze-thaw (pfu at 10⁻⁴)</th>
<th>Not sedimented by 3 min. at 1000 rev/min. (pfu at 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 (1/20) IND</td>
<td>1 (1/30) IND</td>
<td>3·5</td>
<td>130</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>20 'stor' + 7 UVI</td>
<td>1 (1/30) IND</td>
<td>3·5</td>
<td>130</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>45 'stor' + 15 UVI</td>
<td>1 (1/30) IND</td>
<td>3·5</td>
<td>130</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0 (1/20) NJ</td>
<td>1 (1/30) IND</td>
<td>3·5</td>
<td>130</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>45 'stor' + 15 UVI</td>
<td>1 (1/30) IND</td>
<td>3·5</td>
<td>130</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>20 'stor' + 7 UVI</td>
<td>1 (1/30) IND</td>
<td>3·5</td>
<td>130</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>45 'stor' + 15 UVI</td>
<td>0 (1/30) IND</td>
<td>3·5</td>
<td>130</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>20 'stor' + 7 UVI</td>
<td>0 (1/30) IND</td>
<td>3·5</td>
<td>130</td>
<td>65</td>
<td>3</td>
</tr>
</tbody>
</table>
evident in Table 1 among UVI or storage-inactivated particles, as the plating efficiency of the cells receiving inactivated virus (monolayers 7 and 8) in terms of the ratio of cells infected (pfu added): infective centres recovered was the same as the controls (monolayers 1 and 4). No reactivation of the inactivated serotype by the active was detected either (monolayers 3 and 6, columns 9 and 10), in contrast to the findings of Gotlieb & Hirst (1956) with influenza; this may be due to the lack of relationship between the two serotypes found serologically (Brooksby, 1949).

Table 2 shows that homotypic UVI virus still had no excluding action when added up to 30 min. after the active virus.

Heterotypic exclusion by live virus

It does not seem possible to show exclusion by live virus directly for any preparation where the inactivated particles exclude and where a content of less than 50% inactive particles cannot be guaranteed. It is possible that all the reciprocal heterotypic exclusion found in the present work by preparations containing live virus is due to the inactive particles also contained. The most that can be said at present is that wholly inactive preparations behave similarly to preparations containing not more than 50% active particles, the rest being heat- or storage-inactivated (Table 8). As in Table 1, exclusion is reciprocal between NJ and IND, but IND is the more efficient excluding agent. Free and associated infective virus was negligible.

Minimum time to establish exclusion

Table 3 shows that with NJ VS as the primary inoculum and IND as the secondary, addition of the secondary inoculum only 12 min. after the primary still caused a marked decrease in the number of cells releasing IND VS. The significance of this is discussed below.

Likelihood that heterotypic exclusion is reversible

Heterotypic exclusion is not complete: 20-40% of the cells infected by the primary serotype yield virus of the secondary serotype. This indicates either that some cells can support growth of both serotypes at once, or that the process of infection is reversible in many cells at the stage at which they have been challenged.

Table 4 shows the results of infecting cells with both NJ and IND serotypes in such a way that the efficiencies of exclusion were about equal, i.e. the less efficient NJ serotype was present in threefold excess of multiplicity. Significant elution of cell-associated virus from infected cells has never been found in these experiments. The only interpretation of the experiment of Table 4 is, therefore, that the secondary serotype overcame the primary in almost precisely half the cells, while mixed yielders were negligible. Mutual exclusion was demonstrated: a cell was able to liberate VS virus of only one serotype, but the primary infection was reversed in half of the population by the secondary serotype. This was so whether the primary serotype was IND or NJ.
Table 2. Lack of effect on infected cell count of superinfecting with homotypic UVI VS virus (IND serotype)

The virus stock contained $1.8 \times 10^8$ pfu/ml.; the 'active' seed was stock diluted 1/3 (giving multiplicity of infection, M, of 24 when added in 0.5 ml. amounts to monolayers containing $1.25 \times 10^7$ cells), and the UVI seed was stock irradiated to $3 \times 10^8$ pfu/ml. (9 lethal hits, giving M=70). The primary inocula (0.5 ml.) were removed after varying times, plates drained without washing and the secondary inocula (0.5 ml.) were added for a further 90-40 min. The cells from each monolayer were then washed and re-suspended to 1 ml. for assay.

<table>
<thead>
<tr>
<th>Monolayer no.</th>
<th>Primary inocula</th>
<th>Secondary inocula</th>
<th>Total pfu</th>
<th>Not sedimented by 3 min. at 1000 rev/min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>Infectivity</td>
<td>Min. adsorption</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>Active</td>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>Active</td>
<td>14</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>Active</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>Active</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
<td>UVI</td>
<td>34</td>
<td>0</td>
</tr>
</tbody>
</table>

Infective centres per monolayer
Table 3. Reciprocal heterotypic exclusion between live preparations of IND and NJ serotype VS virus, and its apparently rapid onset

The primary inocula (0.5 ml.) were allowed to adsorb for the indicated times, when they were removed and the secondary inocula (0.5 ml.) added for 30 min. The cells from each monolayer were then washed and resuspended in 1 ml. for assay. The NJ stock originally contained $1 \times 10^{9}$ pfu/ml. and had inactivated to $3 \times 10^{6}$ pfu/ml. at $-20^\circ$; it was used undiluted or diluted 1/50. The IND stock originally contained $2 \times 10^{9}$ pfu/ml. and had inactivated to $7 \times 10^{9}$ pfu/ml. at $-20^\circ$; it was used diluted 1/2 or 1/100. 'NJ serotype' and 'IND serotype' represent plaque assays with respectively IND and NJ antisera in the agar overlay. M=multiplicity of infection with live or 'stor' = storage-inactivated ($-20^\circ$) virus.

<table>
<thead>
<tr>
<th>Monolayer no.</th>
<th>Primary inocula</th>
<th>Secondary inocula (M=1(1)), Type</th>
<th>Total cells recovered $\times 10^6$</th>
<th>NJ serotype pfu at $10^{-3}$ per ml. $\times 10^4$</th>
<th>IND serotype pfu at $10^{-3}$ per ml. $\times 10^4$</th>
<th>Infective centres per monolayer</th>
<th>Not sedimented† by 3 min. at 1000 rev./min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>—</td>
<td>50 IND 8-0</td>
<td>45 $(10^{-3})$</td>
<td>—</td>
<td>9-0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>13+</td>
<td>NJ 12</td>
<td>40 'stor'</td>
<td>8-0</td>
<td>70</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>13+</td>
<td>NJ 17</td>
<td>40 'stor'</td>
<td>8-5</td>
<td>78</td>
<td>14</td>
<td>2-6</td>
</tr>
<tr>
<td>4</td>
<td>13+</td>
<td>NJ 25</td>
<td>40 'stor'</td>
<td>8-0</td>
<td>144</td>
<td>12</td>
<td>3-4</td>
</tr>
<tr>
<td>5</td>
<td>13+</td>
<td>NJ 50</td>
<td>40 'stor'</td>
<td>8-0</td>
<td>180</td>
<td>12</td>
<td>2-9</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>—</td>
<td>40 NJ 9-5</td>
<td>72 $(10^{-3})$</td>
<td>14</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>15+</td>
<td>IND 40</td>
<td>90 'stor'</td>
<td>9-0</td>
<td>35</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

* The intact cell preparation from monolayers 5 and 7 gave no plaques when plated at $10^{-3}$ with both antisera.
† Preparations from monolayers 2, 3, 4 and 5 after freeze-thaw or sedimentation and plated at $10^{-3}$ with NJ antiserum, and from monolayer 7 plated with IND antiserum, gave no plaques.
### Table 4. Distribution of serotype among infective centres produced by mixedly infecting monolayers with live IND and NJ serotypes at multiplicities giving approximately equal efficiencies of exclusion

The IND stock initially contained 1.8 × 10⁶ pfu/ml, had inactivated to 3.6 × 10⁶ pfu/ml at –20°C and was used diluted 1/3. The NJ stock initially contained 1.2 × 10⁶ pfu/ml, had inactivated to 6.8 × 10⁶ pfu/ml at –20°C and was diluted 1/2. Primary inocula (0.5 ml) were removed after 30 min., when the secondary inocula (0.5 ml) were added and left for 30 min. The cells from each monolayer were then washed and resuspended to 1 ml for assay. 'NJ serotype' and 'IND serotype' represent plaque assays with respectively IND and NJ antisera in the agar overlay. M = multiplicity of infection with live or 'stor' = storage-inactivated virus.

<table>
<thead>
<tr>
<th>Monolayer no.</th>
<th>Primary inocula</th>
<th>Secondary inocula</th>
<th>Total cells recovered x 10⁴</th>
<th>Infective centres per monolayer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M Type</td>
<td>M Type</td>
<td>Total pfu at 10⁻⁴</td>
<td>NJ serotype pfu at 10⁻⁴</td>
</tr>
<tr>
<td>1</td>
<td>10+ IND</td>
<td>0</td>
<td>202 x 10⁴</td>
<td>3.7 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>40 'stor'</td>
<td></td>
<td>170</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>30+ NJ</td>
<td>0</td>
<td>249 x 10⁴</td>
<td>4.5 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>25 'stor'</td>
<td></td>
<td>205</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>10+ IND</td>
<td>30+ NJ</td>
<td>300 x 10⁴</td>
<td>5.4 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>40 'stor'</td>
<td>25 'stor'</td>
<td>241</td>
<td>115</td>
</tr>
<tr>
<td>4</td>
<td>30+ NJ</td>
<td>10+ IND</td>
<td>200 x 10⁴</td>
<td>4.4 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>25 'stor'</td>
<td>40 'stor'</td>
<td>220</td>
<td>116</td>
</tr>
</tbody>
</table>
Table 5 shows that the efficiency of reversal (proportion of secondary to primary serotypes in the total yield of infective centres) was considerably increased when the multiplicity of infection of the secondary serotype was increased. Free and elutable virus was negligible.

**Minimum number of particles required for heterotypic exclusion**

As the total number of particles (infective plus non-infective) in the VS preparations is not known, and as inactive particles can be shown to exclude heterotypically, any estimate of number of particles required for exclusion must be regarded as a minimum. Table 6 shows that, after primary adsorption of inactivated NJ VS to varying multiplicity, the number of secondary serotype infective centres present in a monolayer after secondary adsorption of about 80 live heterotypic particles (1 per 2 \( \times \) \( 10^5 \) cells) was significantly affected only when the average multiplicity of primary infection was between 1 and 5.

Comparison of these data with those of Baluda (1957), who showed that only one inactivated Newcastle disease virus particle was required to initiate exclusion, strongly suggests that many VS particles are required. This conclusion is supported by the large number of yielders (20–40% of total cells) usually found in cell populations completely infected with a high multiplicity of active or inactive interfering virus, despite a low (single) multiplicity of challenge virus. Since much heat-inactivated virus must also be present the process does not seem very efficient, but adsorption of the higher number of primary particles in Table 6 improved the efficiency, due perhaps to cooperation between particles, or an increased probability that a single particle may succeed.

Plaque size was also markedly reduced at about the inoculum size where most cells had received one inactivated particle, but it is noteworthy that plaques were still able to form when the multiplicity was 50. On these high multiplicity plates the cells between plaques stained well with neutral red and did not differ from controls in microscopical appearance. Thus inactivated virus alone did not harm cells, but in the plaques all cells were unstained and showed much cytopathic effect, so that the initial protection once afforded to at least half the cells had lapsed, either by the passage of time or by reversal by a high multiplicity of infection within the plaque. On the other hand, the fact that plaques were smaller means that interference must in many cells and in some form have lasted at least as long as the time required for infection of the second cycle in the plaques (4–12 hr.).

**Lack of effect of pre-inoculation with inactivated homotypic VS virus on release curves**

Two monolayers were inoculated with a high multiplicity of UVI + storage-inactivated IND VS and a third with medium only as a control. After adsorption, one inactivated-inoculated plate and the control plate were completely infected with a low multiplicity (average = 2) of active homotypic virus, and the other inactivated-inoculated plate had medium only as a second control. After a further adsorption period, the cells were removed with trypsin and
Table 5. Decreasing efficiency of heterotypic live-virus exclusion by the primary inoculum with increasing multiplicity of superinfecting live virus

Virus stock initially contained 1\(\times\)10⁶ pfu/ml (IND) and 1\(\times\)10⁸ pfu/ml (NJ), and had inactivated to 1\(\times\)10⁶ and 1\(\times\)10⁷ pfu/ml respectively on storage at \(-20\)°. The primary inocula (0-5 ml) were removed after 30 min., monolayers washed once and the secondary inocula (0-5 ml) added for a further 30 min. The cells from each monolayer were then washed and resuspended in 1 ml for assay. ‘NJ serotype’ and ‘IND serotype’ represent plaque assays with respectively IND and NJ antisera in the agar overlay; addition of both antisera to plaque assays from monolayer 5 gave no plaques. M=multiplicity of infection with live and ‘stor’=storage-inactivated virus. *=2\(\times\)10⁻⁴.

** Infective centres per monolayer

<table>
<thead>
<tr>
<th>Monolayer no.</th>
<th>Primary inocula</th>
<th>Secondary inocula (IND) M</th>
<th>Total cells recovered (\times)10⁵</th>
<th>Total pfu</th>
<th>NJ serotype pfu</th>
<th>IND serotype pfu</th>
<th>Cells intact</th>
<th>After freeze-thaw, pfu at 10⁻⁴</th>
<th>Not sedimented by 3 min., pfu at 10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1 (1/10)+</td>
<td>1·6</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>7</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1+</td>
<td>1·75</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>25</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>3+</td>
<td>1·48</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>19</td>
<td>37</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>3+</td>
<td>NJ</td>
<td>1·2</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>1·2</td>
<td>1·2</td>
<td>1·2</td>
</tr>
<tr>
<td></td>
<td>30 'stor'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3+</td>
<td>NJ</td>
<td>0·08</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>1·1</td>
<td>0·9</td>
<td>0·075</td>
</tr>
<tr>
<td></td>
<td>30 'stor'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3+</td>
<td>NJ</td>
<td>1·08</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
<td>0·3</td>
<td>0·075</td>
<td>0·075</td>
</tr>
<tr>
<td></td>
<td>30 'stor'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6. *Heterotypic interference in number and size of IND plaques by inactivated NJ VS*

The NJ stock originally contained $2 \times 10^8$ pfu/ml. and fell to $5 \times 10^2$ pfu/ml at $-20^\circ$, and was then ultra-violet irradiated to reduce survivors to a further $10^{-4}$; the average inactivated NJ particle behaved as if it had received $4$ 'storage' + $9$ UV lethal damages. Varying dilutions of this stock were then adsorbed in $0.5$ ml amounts to $8$ monolayers for $1$ hr. at $37^\circ$, three further monolayers having medium only as control. The inoculum was removed, and $0.5$ ml of IND stock, diluted to contain about $80$ pfu, was adsorbed at $37^\circ$ for a further hr., when agar overlay containing NJ antiserum was added.

<table>
<thead>
<tr>
<th>Monolayer no.</th>
<th>Primary inocula (NJ)</th>
<th>Secondary inocula (IND)</th>
<th>Plaques per monolayer</th>
<th>% IND-infected cells yielding progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diln. of stock</td>
<td>Multiplicity of total particles</td>
<td>Approx. pfu added</td>
<td>$&gt;2$ mm. diam.</td>
</tr>
<tr>
<td>1</td>
<td>Neat</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Neat</td>
<td>50</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1/3</td>
<td>16</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1/10</td>
<td>5</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1/30</td>
<td>1.6</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1/100</td>
<td>0.5</td>
<td>80</td>
<td>71</td>
</tr>
<tr>
<td>7</td>
<td>1/300</td>
<td>0.16</td>
<td>80</td>
<td>135</td>
</tr>
<tr>
<td>8</td>
<td>1/1000</td>
<td>0.05</td>
<td>80</td>
<td>79</td>
</tr>
<tr>
<td>9-11</td>
<td>Control</td>
<td>0</td>
<td>80</td>
<td>97, 74, 79</td>
</tr>
</tbody>
</table>
Table 7. **Lack of effect of pre-adsorption of storage-inactivated plus UVI IND VS on plating efficiency after subsequent infection with active IND VS**

The virus stock used initially contained $2 \times 10^5$ pfu/ml and had inactivated to $1.7 \times 10^5$ pfu/ml at $-20^\circ$; part of this stock was irradiated with UV to a survival of $3 \times 10^{-4}$ (5 lethal hits). The primary inocula (0.5 ml) were medium only or UVI stock diluted 1/2; after 1 hr, this was replaced by the secondary inocula (0.5 ml) of medium only or active virus stock diluted 1/5. After a further 30 min, the cells were washed and resuspended to 1 ml for assay. Part of this suspension was diluted in medium to $10^4$ cells/ml for 1-step virus release at $37^\circ$ (Fig. 1). M=multiplicity of infection with UVI, storage-inactivated ('stor') or active virus.

<table>
<thead>
<tr>
<th>Monolayer no.</th>
<th>Primary inocula M</th>
<th>Secondary inocula M</th>
<th>Total cells recovered $\times 10^4$</th>
<th>Total at 0-83 $\times 10^{-4}$ per ml $\times 10^4$</th>
<th>Plating efficiency (%)</th>
<th>Not sedimented by 3 min at 1000 rev./min. at $10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 UVI + 50 'stor'</td>
<td>0</td>
<td>9-5</td>
<td>94, 87</td>
<td>1.8</td>
<td>0.19 (10^{-2})</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>2 active + 8 'stor'</td>
<td>9-25</td>
<td>116</td>
<td>302</td>
<td>32.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>131</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>20 UVI + 50 'stor'</td>
<td>2 active + 8 'stor'</td>
<td>9-75</td>
<td>95</td>
<td>242</td>
<td>24.6</td>
</tr>
</tbody>
</table>

Infected centres per monolayer (pfu)
suspended to $10^4$/ml. in a 50% (v/v) mixture of PBS and 'conditioned' medium for one-step virus release at 37°. Figure 1 shows that this pre-adsorption of inactivated VS had no effect on the release rate or latent period; a slight apparent decrease in final yield was not confirmed in other experiments and is not regarded as significant. As in Table 1 there was also no significant exclusion in terms of number of infective centres recovered (Table 7). In the suspension inoculated with inactivated virus only, the ratio of infective centres recovered to active particles added was if anything slightly less than the plating efficiency of the other cells, 18% compared with 30%, indicating that multiplicity reactivation was not occurring. The release curve of this suspension is less clear in the early stages than the others, but the latent period (3-5 hr.) is not very different from that usual for single infection (4-5 hr.) and the rate of release and final yield are also not much affected, despite the large excess of inactive virus adsorbed within the same 1 hr. period.

**DISCUSSION**

A problem raised by the phenomenon of shortened latency (Cooper, 1958), and the one dealt with in this paper, is whether or not each adsorbing particle of VS virus during more or less simultaneous multiple infection (i.e. during the
Virus exclusion

first half-hour of the latent period), contributes equally to the synthesis of progeny.

Interference and exclusion occur widely among viruses, and the precedent from bacteriophage work suggests that mutual exclusion may occur between unrelated viruses, but related viruses may allow some mixed infection. On the other hand, interference showing as cross-protection is a criterion of relationship among plant viruses. One cannot, therefore, predict from these cases whether exclusion might occur between virus particles of the same or different serotypes of VS virus.

One practical criterion for the problem stated above is the absence or presence of exclusion in the sense of the non-release, in infective form, of a particular genome participating in a mixed infection. Homotypic interference in the sense of depressed rate of release and lower yield by live virus (compared at varying multiplicities of infection) has been shown not to occur with dilute passage stocks of VS virus (Cooper, 1958), although undiluted passage stocks of both VS serotypes contain a non-infective component capable of marked homotypic exclusion (Cooper & Bellett, to be published). The present paper shows that UVI, heat- and 'storage'-inactivated dilute-passage VS virus preparations do not exclude live virus homotypically under conditions where the same inactivated preparations markedly exclude heterotypically. Live virus preparations also exclude heterotypically. The many observations (Luria, 1958) that practically no interfering phenomenon shown by live virus is destroyed by a relatively low number of UV lethal hits makes it seem likely that live VS virus will not exclude homotypically, so that all particles of a mixed infection should contribute equally to progeny. A more definite conclusion for VS virus must await further evidence, perhaps from genetically labelled strains.

Among the many cases of interference by animal viruses, the easiest interpretations, from a cellular viewpoint, involve influenza and Newcastle disease virus (NDV). Like VS, inactivated influenza virus interferes completely and rapidly with heterologous virus (except that only 1 particle may be required per cell (Fazekas de St Groth & Edney, 1952)), but not with homologous virus (save for some depression of yield), provided that super-infection occurs within a few hours (Frazer, 1958). On the other hand, Baluda (1957) showed that UVI NDV excluded homotypically and rapidly, and evidence was given to suggest that the content of non-infectious haemagglutinin was low. However, where 'incomplete' interfering virus may exist and where homotypic exclusion has been found in inactivated virus preparations (unlike dilute-passage VS), further evidence is required to ensure that such exclusion cannot be due to a relatively low content of non-infectious haemagglutinin, e.g. equal in number to infective particles. Baluda also found that, like heterotypic VS, the exclusion in homotypic NDV was to some extent reversed by an increase in multiplicity of the superinfecting virus, although the exclusion appeared much more efficient in the case of NDV in that one particle only was required, whereas VS (heterotypic) probably needs several or very many. VS (homotypic) is even less efficient in that no exclusion was detectable up to
multiplicities of 70; this contrasts markedly with the efficiency of the non-infectious component of VS undiluted passage (Cooper & Bellett, to be published), in which a small number of particles, perhaps one, excluded homotypically.

The presence of marked heterotypic exclusion in VS when the secondary inoculum was added only 12 min. after the primary suggests that the process of establishing exclusion may be quite rapid. It should be made clear, however, that this may not be the case. If the apparent critical time for exclusion of the secondary particle was, for example, 1 min. after the primary, this merely means that the process for irrevocably establishing the secondary infection is shorter by one minute than the process of irrevocably establishing exclusion. Only in the special case where the time for completely establishing the secondary infection is infinitely small can exclusion be said to be established in 1 min. The process could in fact be quite lengthy, and an apparently rapid exclusion indicates rather that the two processes of infection and exclusion are of similar length. The apparent reversibility of exclusion with increasing multiplicity of secondary infection (which may also explain the minority of cells which fail to show heterotypic exclusion) may be explicable in terms of a competition for a particular site or of a change akin to shortened latency in probability or rate of establishment either of infection or of exclusion which is due to the increase in multiplicity of infection.

I am grateful to the staff of the Research Institute, Pirbright, Surrey, for the original virus stocks, and for several generous gifts of high titre specific antisera.

REFERENCES


(Received 9 April 1958)
Tetrazolium Salts as Stains for Animal Virus Plaque Assays

Neutral red (NR) greatly helps plaque counting by contrasting plaques as colorless zones against the red-staining live cells (1). NR is adequate for several systems, but is rather unsatisfactory for agar-suspension plaque assays (2) of poliovirus with the ERK (3) cell line, in which the cells often stain poorly or die with NR, or crystals form in the medium; NR cannot be incorporated in the agar. The staining ability of different NR batches is variable and may be unstable; contrast is decreased by adding a compound which is itself colored. Adding N HCl (Dr. F. K. Sanders, personal communication) or M/2 acetate at pH 4.5 increases contrast, which, however, fades completely within five minutes or an hour, respectively. The untreated stain fades in 1–2 days and is not “fixed” by formalin or ultraviolet light (UV).

Thus our NR-stained plaques are often difficult to count. Triphenyl tetrazolium chloride, a colorless salt reduced inside yeast cells to an insoluble highly colored formozan (4), might give better contrast without fading, but did not stain our cells. Dr. A. G. Everson Pearse (personal communication) suggested that this may be because animal cell redox potentials are relatively high, and that 2-(p-
LETTERS TO THE EDITORS

iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT),¹ having a higher potential, may be more easily reduced.

In practice INT gave good plaque contrast; the red-brown intracellular color required a concentration between 25 and 100 µg INT per milliliter and was maximal after 10-20 hours at 37° and at neutral pH. Routinely we now add 1.0 ml per 65-mm plate of an autoclaved 1.5 mg/ml solution of INT in 0.9% NaCl, and count plaques after 0.5-2 hours at 37°. The minimum cell number per plate (3 to \(4 \times 10^9\)) is the same for NR and INT. With phenol red media, contrast is improved by acidifying after staining.

The advantages of INT over NR for our system are (a) plaque counting is made easier by better color contrast; (b) plating efficiency is increased slightly, probably because more small plaques are seen; (c) contrast has not faded after some months, thus plates can be stained and counted later at convenience or kept for demonstration; (d) plates are fixed with formalin, UV, and acid without fading. Unfortunately, as the insoluble formozan should remain in situ whether the cells die or not, INT seems unsuitable for agar incorporation; agar containing INT (10-200 µg/ml) in fact gave no plaque contrast, although cells were stained and plaques clearly seen by indirect lighting. However, convenience of incorporation of a stain may need to be weighed against the plating efficiency desired (d).

REFERENCES


Virus Culture Laboratory
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Received February 6, 1959

I thank Dr. Everson Pearse for advice and tetrazolium samples.

¹ Lights & Co., Colnbrook, England, supply INT.
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THE CHEMICAL APPROACH TO THE STUDY
OF ANIMAL VIRUS GROWTH

P. D. COOPER

Virus Culture Laboratory, Medical Research Council Laboratories,
Carshalton, Surrey

Abbreviations used in this article are: pfu = plaque forming unit, TCD_{50} = tissue culture dose infective for 50% of the cultures, RNA = ribonucleic acid, DNA = deoxyribonucleic acid, CPE = cytopathogenic effect.

DEFINITION OF SCOPE

There are, broadly speaking, four levels of complexity at which one can study an animal virus infection: (a) the events inside a cell, (b) the events inside an organ, (c) the events inside an animal, (d) the events inside a population of animals. Each has its own importance and information is needed on all for reasons both academic and applied. However, understanding all ultimately depends upon understanding the first. The study of the first in isolation has only recently been technically feasible, and it is an important task of modern virology to emulate the bacteriology of the late nineteenth century in the study of the micro-organism in its simplest environment. Therefore the ‘virus growth’ here given a chemical approach will mean the way in which virus progeny are produced from an infected animal cell.

GENERAL VIEW OF THE PROBLEM

The intricacies of animal virus growth are still hidden from humankind, particularly during the important ‘eclipse’ phase, and a direct way to reveal them is to make the correct chemical analyses. Unfortunately this has proved to be quite difficult, partly because unsuitable systems have prevented strict interpretation (Bauer, 1953); more recently, cell culture has in some degree allowed the use of the fruitful methods familiar to bacteriophage work (in particular the habit of thinking of an animal virus infection in terms of the fate of an individual cell, whatever the size of the population studied).

It is clear, however, from the limited data to hand that such a discipline has not ended our troubles. The animal cell, unlike the bacterial cell, is so large compared with its virus yield that small changes may be masked, and big changes are suspect as they may be unrelated to the primary
growth process. The difficulty in choosing the ‘correct’ chemical analyses then becomes apparent and is still very much with us.

Furthermore, what might be called the overall mechanics of infection are still obscure, and a prior knowledge of them is most important in the design of the most informative chemical experiments. For example, mutual exclusion between virus particles may or may not occur; it is not useful to employ high multiplicities of labelled virus to look for the site of nucleic acid replication if 99% of the label is diverted by genetic exclusion. Chemists might indeed feel that any chemical investigation is premature until the system is better understood biologically.

Happily for us this feeling has not been general, and this reviewer does in fact find himself with material for his review. This contribution will first consider current experience of experimental pitfalls, then review recent work using a chemical approach to simplified cell-virus systems and lastly attempt a synthesis and discuss indications for further work.

SOME FURTHER RESTRICTIONS

It is necessary to restrict somewhat further the scope of this contribution. Cytochemical aspects, including fluorescence and fluorescent-antibody microscopy, are a legitimate if qualitative chemical approach but will be the concern of other contributors to this symposium. Growth-curve studies using serological techniques will also not be included.

Chemical work relating to virus structure \textit{per se}, while very relevant to virus growth, is properly the subject of others and will not be included unless directly contributing to knowledge of the growth cycle. Some effects of inhibitors and substrates on virus growth, perhaps the inverse of the present topic, have been reviewed by Tamm (1958). Studies on the action of chemotherapeutic agents undoubtedly did, or will, give information on the normal growth pathways which they disturb, but experience with the antibiotics suggests that interpretation is a lengthy business. The main lesson from the use of inhibitors so far is that the cell-virus complex has a very similar metabolism to that of the uninfected cell.

The main restrictions will arise, however, because it is not felt useful at this stage to discuss work which cannot give a direct idea of the fate of a single infected cell. It is in any case probable that most current work, even when ideally planned, cannot be interpreted in precise chemical terms, but in some cases it can at least be said that certain things do or do not happen to a cell-virus complex at a particular stage of its development. These can be reproduced in other laboratories, despite small differences in technique leading to perhaps growth cycles of differing length,
and one can hope that such facts will in time build a composite detailed picture. It is therefore disappointing to find that, despite the clear lessons of the last ten years in all aspects of virus work, extensive chemical work is still being done with the unsuitable systems or concepts which were the only ones available ten or twenty years ago. Some reasons for their unsuitability are discussed in the next section.

CRITERIA FOR SUITABILITY OF EXPERIMENTS
It is essential that some at least of the following criteria should be strictly applied to any virus-cell system intended for chemical study. Similar criteria have been discussed by Dulbecco (1955), but with emphasis more on suitability for infectivity than for biochemical studies.

One-step growth
Probably the most important single criterion is that one-step growth conditions must apply. The importance of this is that very many chemical changes must occur in an infected cell as it passes from the normal healthy state to the final stages of lysis and complete disintegration. Thus it is of little interest to record a change unless one can say to what aspect of virus growth it relates. The simplest form of one-step growth system is the single cell, but chemical methods are mostly too insensitive for such very small samples. Furthermore, chemical handling usually destroys the cell-virus complex, so that to cover all stages of infection one needs a number of single cells large enough to overcome their individual asynchrony, which may itself be large. It is therefore easier to use a large cell population in the hope that this represents a fair average, but it is clearly essential that all cells should be at a nearly identical stage of virus development. One-step virus growth per se can be obtained in any cell population if subsequent or previous growth cycles are isolated, for example, by cell dilution, short time-sequences or the use of an antiserum, but chemical studies further demand that one-step growth be applied to the whole culture, as the infected cells cannot be segregated for chemical handling. The metabolism of even a small proportion of healthy cells may mask changes due to virus, and the presence of cells in advanced stages will obscure early changes in later starters. Several authors have taken the trouble to record absence of change in systems where most cells were not infected.

Thus all cells present must be infected at the beginning of the experiment and samples should be taken for chemical assay at intervals during the complete cycle of virus growth (this may not apply to the use of
CHEMISTRY OF ANIMAL VIRUS GROWTH

labelled virus). As we now conceive it, the virus cycle consists of (a) virus adsorption, (b) penetration, (c) growth (of apparently non-infective units), (d) maturation (becoming infective), (e) release.

Examples of systems in which these conditions cannot be fulfilled are intracerebral inoculations, infections of minced-tissue suspensions and of chorio-allantoic membranes in intact or de-embryonated eggs or as in vitro pieces. Allantoic cells in the intact egg (but not as pieces in vitro because of the presence of differently reacting cell types), on the other hand, are suitable for some experiments with labelled virus, although they suffer from the difficulty of disrupting the cells cleanly. Minced tissue is not suitable, owing to the presence of many dead cells.

In vitro versus in vivo

To study a disease itself one must at some stage consider the intact animal, but to study the simplest cell–virus relationships the animal must be eliminated. Use of an intact animal will vitiate all the criteria here proposed, but another reason for avoiding animals is the complication of their hormonal and specific and non-specific defence responses; these need looking at separately.

Virus assay

It is essential to measure infective virus, and not rely solely on other methods of assay such as haemagglutinin titration. The most accurate and sensitive methods applicable should be used and methods such as pock or plaque assays are much preferable to limiting dilution assays. Occasionally one has to choose between an accurate method and a sensitive one, and in this case the best, if perhaps more tiresome, course is to avoid the choice by using both. This is important, as one should define the stage of virus growth precisely; clearly an accurate assay helps this, but one needs to know the largest amount of infective virus involved (for example, to calculate the multiplicity of infection, the end of the latent period or the non-infective particles), so that the titration must also be sensitive. Virus non-infective for one system but infective for another (as detected by systems of different sensitivity) constitutes a form of incomplete virus, which is discussed below as a hazard in interpretation. For these reasons also it is clear that virus must be released into a fluid medium and not extracted in unknown yield from intact tissue.

Early changes the most important

As mentioned above, since the cell eventually dies and disappears almost entirely, there must ultimately be many large changes. Probably the bulk of these can be regarded as remote effects of virus action rather than
effects intimately connected with virus growth and their elucidation may arouse scant interest. It follows that most chemical attention should be given to early phases of the growth cycle, particularly the latent and early release phases.

**Purity and reproducibility of system**

It is self-evident that ideally one should have pure (clone-picked) virus and cell systems, that virus growth curves and assays should be highly reproducible, and that one should be able to take representative samples easily. Unhappily, for many systems this is still easier (or more often) said than done; use of cell suspensions rather than monolayers simplifies the sampling problem, but may increase the proportion of non-viable cells unless adapted to growth in suspension.

**HAZARDS OF INTERPRETATION DUE TO MIXED OR MULTIPLE INFECTION**

The foregoing section describes means of resolving known technical hazards of interpretation. Further difficulties still exist which are only partly understood at present, and which all stem from the likelihood of a cell receiving more than one particle, dead or alive. They may or may not apply to all systems, but when appreciated should be resolvable. Unlike those of the foregoing section, experiments with these pitfalls are likely to be very informative because the growth processes are directly modified rather than merely obscured, but such modifications will be mistaken for the original processes unless they can first be noticed and eliminated.

**Exclusion and interference by inactivated virus particles**

It appears that, like phage, adsorption of a very small number of inactivated Newcastle disease virus particles (Baluda, 1957) can rapidly prevent the growth of identical particles adsorbing subsequently (exclusion). On the other hand, dilute passage stocks of vesicular stomatitis virus (VSV) (Cooper, 1958b) and influenza virus (Frazer, 1953) are free of this difficulty in that homotypic exclusion or interference does not develop in the time usually employed for adsorption. It is clear that if most of added, isotopically labelled virus is ‘excluded’ in this way little information can be obtained regarding transmission of label to progeny, although much information on exclusion mechanisms may result. Similarly, interference without exclusion, as shown by reduced rate of virus release, may occur in a cell multiply infected with live virus, although this does not apply to VSV (Cooper, 1958a); the effect of interference and exclusion on the chemical pathways involved is unknown, as are the pathways themselves.
‘Incomplete’ virus

Apart from the influenza group, several ether-sensitive viruses such as VSV (Cooper & Bellett, 1958), Rift Valley fever virus (Mims, 1956), and western equine encephalomyelitis virus (Chambers, 1957) may give rise to transmissible interfering components, which, in influenza at least, can be labelled ‘incomplete’ virus as they are easily recognized serologically as similar to the infective particle. It should be noted that ‘incomplete’ is not a very satisfactory term, since it has developed a number of different meanings in different hands. The interference of the VSV transmissible interfering component (present in stocks serially passaged undiluted) is manifest largely as exclusion.

Thus the avoidance of multiple infection with live or killed particles is not sufficient to ensure freedom from complications due to exclusion or interference. Like that of influenza virus, the transmissible interfering component of VSV is formed on serial undiluted passage; for viruses other than influenza, however, the dilutions and times of harvest of seed pools are rarely given in published articles on virus growth.

Two special difficulties which are similar are (a) the excess of non-infective over infective particles present in, for example, poliovirus (Schwerdt & Fogh, 1957), and (b) differing sensitivities of two assay systems, for example, HeLa cells and chorio-allantoic membranes for herpesvirus (Newton & Stoker, 1958). In both cases fully or partly (that is, for one cell type only) inactivated virus may be present, or all particles may be potentially infective but with a low or variable probability of achieving infection. In the latter case it may be that the probability of interference is higher than that of infection, in which case the excess of particles will function as ‘incomplete’.

Interferon

The discovery of a viral product which is unlike a virus particle and which interferes with virus growth (Isaacs & Lindenmann, 1957) means that complications may exist even with a single multiplicity of ‘complete’ virus fully infective for the system used. Here, as with ‘incomplete’ VSV, one does not have as simple a serological guide as with incomplete influenza virus; thus the need for purified virus as an inoculum becomes apparent and this need has been almost entirely overlooked so far. Some other virus preparations also contain ‘toxic’ components (Pereira & Kelly, 1957; Ackermann, Payne & Kurtz, 1958).
Shortened latency

In some viruses (Cooper, 1958a), increasing the multiplicity of infection decreased the time before appearance of the first progeny particle; that is, shortened the latent period. Two possible explanations among others are that virus grows as a pool equally accessible to all adsorbing virus, and/or that there is a probability of delay in starting an infection which becomes less the more particles are added. Whatever the explanation, there is no guarantee that the chemical changes, particularly the early ones, will be the same for single and multiple infection.

Lysis-from-without

Infected animal cells do not ‘burst’ in quite the same way as bacteria and such a mechanism for releasing virus from enclosure within a thick rigid cell wall is not necessary. Poliovirus does not appear prematurely to damage monkey kidney cells up to a multiplicity of 1000 particles per cell (Fogh, 1955). Nevertheless, possible damage should be sought when contemplating experiments with very high multiplicities.

Multiplicity reactivation

Multiplicity reactivation is not noticeable in u.v.- or heat-inactivated VSV preparations (Cooper, 1958a, b), although marker recombination has been found in inactivated influenza virus preparations (Gotlieb & Hirst, 1956). The complication may arise in, for example, experiments with isotopically labelled virus, where some cells receive a single live virus particle and others receive several inactive particles; the chemical mechanisms of producing progeny may be different in the different cells, again particularly in the early stages.

HAZARDS OF INTERPRETATION DUE TO PROBABILITY EFFECTS

Due to the Poisson distribution of adsorbed virus, and the likelihood that even virus particles simultaneously adsorbed at the same multiplicity may not start growing at the same time, it is likely that the chemical changes in a population of multiply-infected cells will be neither synchronous nor identical. The presence of shortened latency makes such asynchrony inevitable for VSV (Cooper, 1957a, 1958a), and this difficulty seems unavoidable where one insists on multiple infection yet cannot handle a single cell.

The only recourse seems to be to say that this may not matter very much providing the discrepancies are small or cancel one another, so
that one can observe an average (although an average of what is open to question). The greatest difficulty is to decide whether to ascribe a small effect to a small change in all the cells or to a big change in some, perhaps in those few which happen to be very advanced in lysis, that is at a stage less relevant to the main mechanisms of growth.

THE IDEAL EXPERIMENT

To illustrate all these difficulties it may be helpful to construct an ideal experiment as a basis for following the chemical changes accompanying virus growth in animal cells. Leaving aside the beautiful but unrealizable concept of performing successive quantitative chemical assays on the same single cell throughout a growth cycle, we are left with the usual cell population.

The experiment should run as follows: the cells are first derived by a minimum number of subcultures from a single cell, and maintained frozen as a master culture. Thus cells for all experiments have the same history, as they are derived from this master. A thick agitated suspension of such cells in buffered saline is completely infected with virus under conditions where adsorption is complete before growth begins. The virus seed has been derived from a single plaque, has been maintained by a minimal number of dilute-passage subcultures from a master stock, and is free of incomplete, inactivated and interfering particles; 1 infectious unit = 1 electron-microscopic particle. The virus is completely stable at 37°, and the seeds used have been extensively purified chemically and physically before use. Since the cells are also growing systems, their growth processes have been synchronized or halted, but 100% of the cells present are still viable, or at least able to liberate the maximum yield of virus. Surface damage, by, for example, trypsin or versene, has been overcome before the experiment is started. After adsorption, free unadsorbed virus is eliminated, and virus growth started in all cells at once, still in suspension in a completely defined synthetic medium and under conditions where virus readsoption does not occur. After a short time for penetration, adsorbed virus which has not penetrated is also eliminated. Samples are then taken for total-cell, infected-cell, and free-virus assays. At intervals during the entire growth cycle samples are removed for chemical and infectious unit assays in both cell and supernatant fractions; infectious units are measured at once by plaque assay in the most sensitive system available. Virus growth is rapid and yield per cell is high (for example, 1000 pfu/cell).

The multiplicity and state of infecting virus, etc., can of course be
varied, inhibitors can be used to detect different stages, and the possibilities for chemical and other assays are large; clearly the more different are the assays made on the same system under different conditions the more detailed can be the conclusions.

The results are then interpreted in terms of the events occurring during virus adsorption, penetration, organization into a replicating form, vegetative growth, maturation and release or other stages detected, and correlated with existing information on virus structure (for example, RNA or DNA content, number and nature of lipid or protein subunits) and with information from cytochemical studies, for which monolayer cultures may be more suitable.

Although this form of presentation is necessarily somewhat naïve, it should suffice to show that no published experiments are ideal, and that the obstacles to such an ideal are formidable. It is also little consolation to the animal virologist to know that the bacterial virologist has had almost such a system available with much less effort for many years; consequently much of the preceding sections will surprise phage workers by stating the obvious yet again. Nevertheless, technical difficulties are still so considerable for the animal virologist that the obvious needs restating.

WHERE TO LOOK

One further source of difficulty needs mention. It derives from the fact that the animal cell is a relatively large and complicated structure, containing at least four 'compartments' (namely, nucleus, mitochondria, microsomes, soluble fraction), some of them with double membranes and nearly all themselves highly complex structurally when observed under the electron microscope. When considering that each structure must contain extremely complex chemical pathways, one might be excused some despair of being able to unravel anything useful. Our methods of dissecting cells for examination are still very crude, and methods for separating the contained chemical fractions not much better.

Clearly one needs some sort of a lead, for example from the early fate of labelled virus RNA, or early cytological changes. It is not certain whether cell-virus systems showing large early changes are better than those showing small ones, since the small ones may be the more simple to follow. The writer is inclined to feel that a good approach to this problem will not be apparent until we have better techniques and more information on the general behaviour of the system (for example, whether the nucleic acid of the particular virus is synthesized in the nucleus and the protein in the cytoplasm).
CURRENT INFORMATION ON PARTICULAR VIRUSES

In such an early stage, it is inevitable that chemical information on intracellular growth mechanisms will come to hand in a piecemeal fashion. So as to derive as much order as possible, work which can definitely be interpreted in terms of happenings at the cellular level will be summarized below under the heading of the individual virus.

**Poliovirus**

Several very marked changes have been observed during poliovirus growth. Maassab, Loh & Ackermann (1957) studied one-step growth of poliovirus type 1 (Mahoney) in HeLa cell cultures using sufficient virus to infect all cells initially, and followed the phosphate changes in DNA and in cytoplasmic and nuclear RNA. Starting within an hour of infection the total cytoplasmic RNA phosphate increased 2-3-fold over control values. This was substantiated by a simultaneous increase in the rate of cytoplasmic RNA $^{32}$P incorporation, which continued up to 7 hr., and then declined, whilst the first infective virus particle appeared in the ‘average’ cell at about 3 hr. Thus virus maturation was preceded by a large synthesis of cytoplasmic RNA, at least some of the phosphorus coming from inorganic phosphate. The interest of this finding is enhanced by the fact that poliovirus contains RNA rather than DNA (Schwerdt & Schaffer, 1956). There was also a simultaneous but smaller incorporation into DNA and nuclear RNA.

More recently, Ackermann (1958) has found that the base ratios of the newly formed cytoplasmic RNA are very similar to those of uninfected cytoplasm and quite distinct from those of the virus, so that this does not seem to be a cytoplasmic accumulation of virus RNA. There was a large early increase in protein.

The further interpretation of these findings runs into the difficulties encountered by Cooper (1957a, b). For example, in the poliovirus studies, cytoplasmic ‘RNA’ phosphate was separated by the Schneider method, and no attempt was recorded of a separation from phosphoprotein and hot-acid-labile phosphates which occur in significant amount in animal cells. Also, separation of nuclei may extract loosely bound components so that more than one cell-fractionation procedure is desirable; the cytoplasm itself contains many particulate and soluble components. Smaller but still significant changes also occurred in the nucleus, and it is possible for an animal virus—for example, that of vesicular stomatitis (Cooper, 1957b)—to grow well with much smaller chemical changes than those of poliovirus. Thus one cannot decide from
these results whether or not poliovirus RNA is replicated in the nucleus or the cytoplasm. Nevertheless, the presence of such large and early changes makes poliovirus in cell culture seem an interesting system for further study.

Miroff, Cornatzer & Fischer (1957) have also investigated the uptake of $^{32}P$ into various phosphate fractions of the HeLa cell in monolayer culture during infection with type 1 (Mahoney) poliovirus. The inoculum was insufficient to infect more than 30% of the cells initially, but the $^{32}P$ uptake of the uninfected cells was sufficiently low (perhaps because a non-growth-producing maintenance medium was used) to detect changes from control cell values during the early part of the first cycle (up to 4 hr. after infection). In support of the findings of Maassab et al. (1957), there was an increase in specific activity of total nucleic acid (Schneider separation) and phospholipid fractions. Virus yields appeared atypically low, however (less than 10 TCD$_{50}$ per cell after a full cycle): methods of preparing seed pools were not given.

On the other hand, Goldfine, Koppelman & Evans (1958) found that, during one-step growth of poliovirus type 3 in HeLa cells completely infected with a high multiplicity, the rate of incorporation of cytidine $^{14}C$ into RNA was slightly less than in the controls during the first half of the cycle (up to 5 hr. after infection), returning to normal later (up to 11 hr.). This may indicate that the rate of de novo synthesis of bases was increased.

The incorporation into DNA was greatly decreased during this time.

Some information is also available, in general terms, of the effect of poliovirus infection on the metabolism of small molecules. Levy & Baron (1957) compared glycolysis and uptake of $^{14}C$ glycine by monkey kidney cell monolayers untreated and infected with type 3 poliovirus at a multiplicity of 70. Lactic acid production was noticeably faster by the first hour after infection in this one-step growth system, and the high rate continued until the 7th hour, when it fell to control values. The effect was also found with partially purified virus. The virus stimulation occurred with anaerobic as well as aerobic glycolysis, so that it was not due simply to blockage of oxygen pathways; infected cells also showed the Pasteur effect. Therefore presumably the changes represent an overall increase of glucose utilization, rather than a switch from one pathway to another. On the other hand, glycine uptake was much slowed by infection, being manifest by the first hour, and continuing all through the cycle.

Becker, Grossowicz & Bernkopf (1958) found very similar changes in the uptake of glucose during one-step growth of type 2 (MEF$_2$) poliovirus on human amnion or monkey kidney cell monolayers. Sufficient
virus was added to infect all cells initially (multiplicity = 5), and in comparison with identically treated uninfected cells the infected cells used very much more glucose during the period immediately preceding the bulk of virus release (0–10 hr. after infection). The rate of glucose uptake later fell to control values. The rate of phosphate uptake was also higher in the first 10 hr., but the phosphorus that accumulated was soon afterwards released into the medium. Cyanide, azide and fluoroacetate increased the glucose utilization of infected cells even more than that of controls, without affecting virus yields. It seems, therefore, that anaerobic glycolytic pathways can be used for virus synthesis, but the oxidative ones also play a part.

This conclusion is also derived from the observation that poliovirus grew in the apparent absence of oxygen, but the provision of oxygen as air greatly increased the virus growth rate (Gifford & Syverton, 1957). It was noted that the cells (HeLa and monkey kidney) behaved like facultative anaerobes in the absence of virus.

Ackermann, Rabson & Kurtz (1954) found that p-fluorophenylalanine inhibited viral synthesis during one-step poliovirus growth in HeLa cell monolayer cultures. The inhibition was reversed by phenylalanine, provided this was added within 6 hr. of adding the inhibitor. Although virus growth was stopped, CPE was not. Eagle & Habel (1956) found that salts, dialysed serum, glucose and glutamine were the only nutriments necessary for maximum virus yields, but glucose could be largely replaced by a group of other nutriments added together. Some interesting observations of considerable significance to pathogenicity studies, but of as yet unknown significance to growth studies, are the growth requirements of some strains for the bicarbonate ion (Vogt, Dulbecco & Wenner, 1957) and cystine (Dubes, 1956).

Kovacs (1956a, b) has presented much data on the effect of poliovirus in vitro on various nucleases and phosphatases, mostly using monkey kidney cells in roller-tube culture. Although most of the changes are decreases, many appear before CPE and may therefore have a bearing on processes of virus growth rather than cell lysis, but since one-step growth was not used, the stage of the growth cycle at which changes commence is unknown.

In summary, therefore, poliovirus-infected cells show a generalized, enhanced metabolism in the eclipse period in which one might expect virus synthesis to be most active. These changes were largely cytoplasmic. The cytological changes which are also apparent at this time are largely nuclear (Dunnebacke, 1956).
Adenovirus

Fisher & Ginsberg (1957) investigated the reasons for the more acid appearance of HeLa cell cultures infected with type 4 adenovirus. They found that this was due to stimulation of glycolysis resulting in a higher production of non-volatile acids (lactic, pyruvic, acetic and α-ketoglutaric). In the case of lactate, at least, the proportion of glucose converted to non-volatile acid was unchanged by infection, so that, as with poliovirus, the overall utilization of glucose was increased 3–5 fold during the complete period of virus growth without any overt rearrangement of pathways. One-step growth conditions were not used, however, so that one cannot say at which stage the increase occurred; the authors point out that it cannot be said whether or not increased glucose utilization is an inherent part of adenovirus growth.

Levy and co-workers (1957) described several metabolic changes during one-step growth of adenovirus type 2 in HeLa cells. All cells were infected initially, and the changes were not due to the component giving early CPE, but to the virus itself; adenovirus, however, may be a difficult biochemical subject because of a high degree of asynchrony during infection. Levy et al. found within 2–4 hr. a great increase in the incorporation rate of 32P phosphate into all phosphate fractions without any effect on their total concentrations, and of glycine-2-14C into the acid-soluble fraction. They also found an increase in lactic acid production, but were able to show that this occurred much later (8–24 hr. after infection).

Newcastle disease virus (NDV)

Franklin, Rubin & Davis (1957) prepared purified NDV containing 32P. They found that while some acid-soluble phosphorus could be lost without loss of infectivity, the phospholipid could not, even if removed enzymically. Rubin & Franklin (1957) used this labelled virus to show that most virus particles neutralized by adsorbing a very few antibody molecules could adsorb to chick lung epithelial cells in vitro without entering them. They felt therefore that ‘viropexis’ (engulfment by the cell) could only account for a small proportion of successful viral penetrations, and that the usual mechanism was more rapid and specific, perhaps enzymic.

Vesicular stomatitis virus (VSV)

Cooper (1957b) compared the paths of phosphate transfer in normal chick embryo cell monolayer cultures with those completely infected with VSV under conditions of one-step growth. In view of the small changes found, some pains were taken to ensure that these conditions
did in fact apply, and that substantially all cells were able to liberate good yields of virus (Cooper, 1955, 1957a); however, purified virus seeds could not be used. The rates of transfer (gain, loss or exchange) of $^{32}P$ between acid-soluble inorganic and organic phosphates, lipid, RNA and DNA phosphates, were all unaffected until uptake ceased in most fractions soon after the end of the latent period; the only other change was a 30–50% decrease in sucrose-soluble RNA towards the end of the exponential release, which may be the first stage of lysis in a minority of early-starting cells. Cell particulates appeared undamaged by this time, although at 20 hr., when CPE was extensive, most of the cellular $^{32}P$ from all fractions was released into the medium. Thus the early changes due to virus synthesis must be quite small; those found indicated an overall inhibition of metabolism. Meanwhile Turco (1959), concurrently using the same system in the same laboratory, found the same in general but also a small but significant change in the RNA base ratios of the nucleus, the mitochondria-plus-microsomes and of the sucrose-soluble fraction. The relative proportion of uridylic acid increased; in $^{32}P$ gain' experiments ($^{32}P$ moving into the cells) the relative specific activity of uridylic acid dropped progressively, but in $^{32}P$ loss' experiments it rose slightly. The other nucleotides were unaffected. This suggested the coincidental synthesis of virus nucleic acid and a copious new high-uridine RNA, with equivalent loss of old RNA; the new RNA, although of unknown relation to virus material, was equivalent to very much more than the infective virus yield.

Evidence was also presented (Cooper, 1957c) that the cells possessed a phosphate-impermeable membrane which allowed reciprocal transport of phosphate by presumably specific mechanisms. The rate of phosphate exchange across this membrane was used to show that VSV could enter, grow within, and leave the host cell without grossly damaging the cell surface.

**Fowl plague virus**

Wecker & Schäfer (1957b) completely infected chick embryo cells in monolayer culture with a low multiplicity (1–2) of purified fowl plague virus labelled with $^{32}P$ (Wecker & Schäfer, 1956). Chorio-allantoic membrane pieces were treated similarly. The virus was allowed to grow for periods up to 3 hr., when the cells were harvested, washed and homogenized. Between one-third and one-half of the $^{32}P$ was then extractable with water, the rest being firmly bound to the cell debris. Of the extract, nearly one-half was not sedimented by centrifugation sufficient to sediment intact virus; some of this ‘soluble’ material was precipitated by antisera to *gebundenes antigen* (which can be obtained from purified
intact virus by ether treatment, Schäfer, 1957). The remaining $^{32}$P was in phospholipid, acid-soluble phosphate and free RNA (that is, lysed by ribonuclease).

Thus on infection some fowl plague virus seemed to break down to gebundenes antigen, plus smaller-molecule phosphates and soluble RNA which may or may not be derived from gebundenes antigen. However, most of the $^{32}$P remained attached to the cell debris; also the haemagglutinin: pfu ratios (Wecker & Schäfer, 1956) of the purified virus seed suggest the presence of a large excess of non-infectious haemagglutinin, so that these very interesting results must be interpreted with caution at present.

The virus particle, 70 m$m$ diameter, is composed of lipid and two particulate protein-containing components, the haemagglutinin (HA), 30 m$m$ diameter, and the gebundenes antigen, 10–15 m$m$ diameter, which latter also contains the bulk of the RNA (Schäfer, 1957). Franklin (1958) compared the effect of proflavine on the intracellular appearance of infective virus, the soluble antigen (which closely resembles gebundenes antigen) and the HA of fowl plague virus, in chick embryo cell monolayers. He found that HA and virus synthesis were inhibited at much lower proflavine doses than was soluble antigen, and the former showed much more nearly ‘single-hit’ exponential dose-response relationships than the latter. Soluble antigen appeared first in the nucleus, whereas HA appeared first in the cytoplasm (Breitenfeld & Schäfer, 1957; Franklin, 1958), and several papers were quoted which suggested that proflavine will inhibit cytoplasmic more than nuclear syntheses.

Influenza virus

Hoyle & Frisch-Niggemeyer (1955) examined the fate of purified $^{32}$P-labelled influenza virus after infection of the allantoic sac in ovo. After 1½ hr. when about half the radioactivity was adsorbed to the cells, part of the $^{32}$P recovered from the disintegrated membranes was found, as in the case of fowl plague virus, to be in material of smaller particle size than the intact virus. The remainder was attached to the cell debris, but was eluted with molar sodium chloride. The fate of the RNA $^{32}$P was not clear-cut and probably represented a mixture of fates; little further can be deduced since the chemical and physical separation methods employed would not rigorously define the various fractions obtained.

Again, a difficulty of these experiments is the possible presence of non-infectious haemagglutinin and the need to use large multiplicities of infection in order to detect the small quantities of label, although homotypic exclusion may not be important in influenza virus infections.
A further snag which seems inherent with the myxovirus group, and possibly with all ether-sensitive viruses, is the presence of large amounts of phospholipid $^{32}$P which may be rapidly broken down in the cell and may enter the metabolic pool, thus obscuring the fate of the labelled virus nucleic acid.

Hoyle & Finter (1957) have similarly examined the fate of influenza virus labelled with $^{35}$S-methionine. Their results also indicated a minor but rapid change of physical or chemical state. At least 20% of the protein $^{35}$S changed to small molecular weight material within $1\frac{1}{2}$ hr., and the rest became associated with some poorly soluble component. All haemagglutinin and 98% of the infectivity was lost.

One can therefore say that some of the invading virus particles were broken down to smaller pieces, but the fate of the remainder is unknown. Which group leads to infective progeny is also unknown.

Liu (1956) also found that, of the purified $^{32}$P-labelled influenza virus which adsorbed to the cells of the allantoic sac, some alcohol-soluble $^{32}$P appeared to be transferred to the cold acid-soluble fraction within 6 hr., but the hot acid-soluble fraction did not change. However, nearly all of the virus $^{32}$P added to the eggs appeared unchanged but did not adsorb, although most of the haemagglutinin and infectivity was lost; perhaps this was due to non-specific virus inhibitors which may also have affected the adsorbed virus.

Henle, Girardi & Henle (1955) used the incorporation of $^{32}$P to show that the small amounts of haemagglutinin found in HeLa cell cultures after influenza virus infection was not residual inoculum but did in fact arise from de novo production of a form of incomplete virus. The cells were destroyed, despite the fact that little infective virus was produced.

Ackermann & Maassab (1955) were able to indicate two stages of influenza virus development, the earlier of the two inhibited by methoxinine and the later by $p$-fluorophenylalanine. Between 4 and 6 hr. after infection virus synthesis appeared to pass through a stage after which it was insensitive to both inhibitors.

An observation (Le Clerc, 1957; Burnet, Lind & Perry, 1957) which may have important implications is that influenza virus infections pass through a stage early in the latent period in which they are relatively sensitive to ribonuclease; influenza virus contains RNA, which seems likely to be responsible for the infectivity (Ada & Perry, 1956).

**Herpes virus**

Newton & Stoker (1958) followed chemically the change in nucleic acid content during one-step growth of herpes virus in HeLa cell monolayer
cultures, when all cells were infected initially. In comparison with controls they found a marked increase of DNA per cell which started well within the eclipse phase. Even after 72 hr., when the DNA per cell was doubled, all DNA was confined to the nucleus. The total DNA produced was very much larger than might be accounted for by infective virus progeny, and it would be interesting to compare its base ratios with those of host cell and virus. RNA was not affected. Electron microscopical and fluorescent-antibody studies in the literature were quoted which also suggest that this virus is synthesized in the nucleus.

SOME GENERALIZATIONS

Viewed in the light of the disciplinary restrictions which are discussed in the preliminary sections, the foregoing summaries show that we have, as yet, little detailed chemical information on the intracellular growth of any one animal virus. Nevertheless, certain findings, when coupled with measurements of infectivity, may permit some generalizations.

A most important property which now gives good promise of being general among RNA viruses is that infective material can be extracted in the form of free RNA rather than necessarily bound as virus ribonucleoprotein. The viruses for which this information is now available are those of Mengo encephalitis (Colter, Bird & Brown, 1957), poliomyelitis type 2 and West Nile encephalitis (Colter, Bird, Moyer & Brown, 1957), eastern equine encephalomyelitis (Wecker & Schäfer, 1957a), poliomyelitis type 1 (Alexander et al. 1957), Semliki forest (Cheng, 1958), foot-and-mouth disease (Brown, Sellers & Stewart, 1958), and encephalomyocarditis (EMC) (Huppert & Sanders, 1958).

In general the RNA preparations were more infective in vivo than in vitro, and some question of cellular 'competence' may be involved. Several criteria have been held to show that the infectivity is in fact due to free RNA, but Huppert & Sanders (1958) have shown that the only reliable criterion of this is its sensitivity to ribonuclease, the other criteria at best showing only that the infectivity behaves somewhat differently from intact virus at comparable infectivity concentrations.

An interesting outcome of the work with EMC virus is that the extraction procedure used did not yield infective RNA from purified EMC virus per se but only from a component of infected cells or crude lysates sedimenting more slowly than virus and which was resistant to ribonuclease. Infective RNA of the other viruses was all extracted from cell homogenates or crude or partially purified lysates. One's thoughts turn in this connection to the 'soluble antigen', a virus-specific micro-
some-like small particle component (7–15 m\(\mu\)) of unknown function nearly universally present in cells infected with the smaller animal viruses, and, in the case of fowl plague virus at least, containing RNA. However, its relationship to the small-particle source of infectious RNA remains speculative for the moment.

The lack of penetration of antibody-neutralized NDV (Rubin & Franklin, 1957) suggests that the impetus to penetration comes from the virus rather than the cell, and this is supported by the findings of Allison (personal communication). He found that pretreatment of isotope-labelled fowl plague virus with \(p\)-chloromercuribenzoate (PCMB) or diisopropyl fluorophosphonate (DFP) did not alter the kinetics of virus adsorption but prevented penetration (the PCMB effect was reversed by cysteine). The same applied to vaccinia virus except that DFP did not prevent penetration. Possibly the adsorption sites on the virus surface which lead to successful penetration are sterically blocked by inhibitor and are few compared with those allowing non-infective adsorption, but it is more likely that penetration is mediated by virus enzymes, as DFP is a potent anti-esterase and PCMB inhibits sulphydryl-activated enzymes.

Franklin (1958a) has pointed out the relationships existing between the ether sensitivity of a number of viruses and the way in which they are released from infected cells. Certain ether-sensitive viruses—for example, western equine encephalomyelitis (Rubin, Hotchin & Baluda, 1955) and VSV (Franklin, 1958c)—remain associated with the cell for only a few minutes after maturation, and if VSV (Cooper, 1957c) is typical, are released by a ‘secretory’ mechanism rather than a ‘burst’. Franklin makes the interesting suggestion that all ether-sensitive viruses are completed at the cell periphery with a lipid coat which is essential for infectivity. Removal of this lipid by lipid solvents or enzymes destroys the infectivity of NDV (Franklin, Rubin & Davis, 1957). Viruses such as poliovirus in which lipid is absent, or others in which it is inaccessible (not as a coat?) and so are ether-resistant, are suggested to be not dependent on the cell surface for maturation and in fact accumulate inside the cell until released by a burst-like process (Howes & Melnick, 1957). The sensitivity to deoxycholate of a number of viruses (Theiler, 1957) also seems to parallel closely their ether sensitivity; since by definition ‘enteroviruses’ need to be bile-resistant, this particular classification of viruses according to their habitat may have some foundation in chemical structure. Dulbecco & Vogt (1955) noticed a marked difference between the sensitivities to nitrogen mustard of poliovirus on the one hand, and a group consisting of VSV, NDV and western equine encephalomyelitis...
virus on the other, and this difference may also be related to their lipid content.

Another generalization of relevance to growth mechanisms and based on chemical data has been summarized by Frisch-Niggemeyer (1956). It is that while size, and therefore particle weight, vary widely among the smaller viruses (both plant and animal but all RNA-containing), the content of RNA is approximately constant, suggesting that the fundamental replicating unit is similar. In spite of this, however, the sensitivity to u.v. light is much lower (smaller target?) for poliovirus (Dulbecco & Vogt, 1955, 37% survival dose = 360 ergs mm.\(^2\)) which is 27 m\(\mu\) diameter, than for VSV (Cooper & Bellett, unpublished data, 37% survival dose = 50 ergs mm.\(^2\)) which is 70–80 m\(\mu\) diameter. There also appears to be a grouping of the weights of these viruses around values which are small integral multiples of a smaller subunit (Poison, 1953); this might suggest a common simple assembly pattern from similarly constituted subunits. However, interpretation of this must await further data on size from other methods (for example, electron microscopy) and on chemical composition; some of the viruses may contain lipid, others do not. There is also evidence that individual viruses can exist as particles falling in more than one of these size-groupings.

We might summarize our present knowledge of the 'typical growth cycle' by the following examples from specific viruses. Like phages, animal viruses appear first to adsorb to cells by electrostatic mechanisms (for example, poliovirus; Bachtold, Bubel & Gebhardt, 1957), become rapidly fixed and then penetrate the cell membrane by the action of viral enzymes rather than by phagocytosis by the cell (for example, fowl plague, NDV and vaccinia viruses). Infectivity is now no longer detectable inside most cells later releasing infective virus, so that an eclipse phase in this sense seems well established for all the smaller viruses examined.

Unfortunately we cannot yet say from the chemical data that the eclipse is due to a physical breakdown of the particle. That some degree of breakdown and subsequent assembly of new particles does occur, however, seems likely from the separate synthesis of fowl plague soluble antigen (in the nucleus) and haemagglutinin (in the cytoplasm). Free RNA can certainly play a part in the infective process, but it is not known whether a normal infection with intact virus must necessarily pass through a stage in which the nucleic acid is in free state.

The eclipse phase of poliovirus and adenovirus is accompanied by increased chemical activity in both nucleus and cytoplasm, particularly marked in the latter, which appears at present to be an all-round increase of the normal phosphate and carbohydrate metabolism rather than a
selective change. Herpesvirus causes an increase in nuclear DNA during the eclipse phase. On the other hand, VSV probably produces as much virus nucleic acid in the form of infective particles as does poliovirus, but brings about no gross disturbance of phosphate metabolism in the eclipse phase. Cells appear damaged at similar times after infection with poliovirus or VSV, and big changes are to be expected and may be found in both systems at these times.

Finally poliovirus (and a number of other ether-insensitive viruses) are completed and accumulate within the cell until released by partial cell dissolution, whereas VSV (and a number of other ether-sensitive viruses) appear to be completed at the cell periphery and released at once, and therefore continuously, by a secretory-like process. It remains to be seen whether large and small changes are typical of the two types of maturation.

CONCLUSIONS

Virus synthesis can only be conceived as proceeding in stages, and it is likely that the initiation of the transition from one stage to another is to some extent a matter of chance. If this is so, the operation of chance in the earlier stages, particularly while the virus is organizing into a replicating state and before any replication has begun, will mean that some cell-virus complexes will be more delayed than others in starting to replicate, and the overall chance of delay will increase with the number of preliminary stages. Cairns (1957) has found evidence of some delay in completing the first cycle of influenza virus infections (although he maintains an open mind about which stage in the growth process it occupies), and shortened latency (Cooper, 1958a) could be accounted for partly in this way, although multiple contributions to a vegetative pool seem more likely as an explanation (unpublished considerations). However, if a vegetative pool is formed during replication, the operation of chance will increasingly allow units to be drawn at random from it into another partly-assembled-particle pool, and samples may be drawn at random from such a pool into a matured pool and thence released. Thus, after the early stages, the probability of delay in passing from one stage to another should express itself in the relative sizes of the different pools rather than in a further distribution in time at which virus release takes place. In other words, the time at which individual particles start to replicate may vary widely, but once started the rates of development of individual cell-virus complexes should be similar.

This picture is, of course, speculative, but the point in describing it is that in the infected cell at the height of virus synthesis there may be quite
large co-existing pools of virus components or precursors of all stages of development, increasing at earlier times but constant in amount later, and for a time at least bearing a constant relation to each other. The pools may be much larger than the amount of virus produced if the probability of correct (infective) assembly is low, and this probability may be reflected in the difference in infective yield per unit cell mass between animal viruses on one hand and plant and bacterial viruses on the other. There is indeed some evidence that the scale of the infective processes is similar for all three virus groups; this, if true, would be a useful unifying concept.

Thus, one important step is to find the number of stages and pools involved, particularly the early ones; their nature can be investigated subsequently. If their presence can be established by some means, perhaps serological, use of isotopically labelled components may reveal the sequence in which they are started or assembled, and their age.

There is thus much to be done in deciphering the mechanisms of virus growth; as with phage, probably much is to be gained by concentration on a few well-chosen model systems. Animal viruses are more diverse than bacterial ones, and so more model systems may be desirable. The difficulty remains of making the choice so that the ideal criteria apply as much as possible, and the present phase seems likely to be an exploratory one in which the relationships among the viruses become better known, and more information becomes available on which to base our choice.

I wish to thank all those who were kind enough to show me their manuscripts and data in advance of publication.

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LARGE-SCALE TISSUE CULTURE (P. D. Cooper, B.Sc., Ph.D.)
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'LARGE-SCALE' tissue culture has in the last decade become feasible in considerable measure because of the impetus derived from poliomyelitis virus vaccine production, and doubtless this new field of industrial enterprise will continue to grow, as its commercial potentialities are world-wide. However, tissue culture on a scale even approaching modern full- or pilot-plant-scale operation is so recent, and differs sufficiently from usual microbiological processes, that the trends cannot well be discussed without some introduction to the methods of tissue culture. The differences fortunately are not fundamental but quantitative, as animal cells in the free-living state behave as true micro-organisms.

Definitions
'Tissue culture' implies the growth or maintenance of cells derived in the first place from an animal (nearly always a vertebrate) or a plant, in a living form apart from the organism of origin. Its history for tissue pieces extends back to the early 20th century. However, 'tissue' is nowadays often a misnomer, since the tissue as a whole is not kept, but only certain cells from it; hence strictly speaking one should refer to 'cell culture' or 'organ culture', although 'tissue culture' is still frequently used. While considerable data are now available for cell culture, organ culture is still in its infancy and will not be dealt with here; generally speaking organs can be maintained but not grown in culture, and the first problem to be solved is to prevent the organ from disintegrating by the migration and dispersal of its cells in culture.

Principles of cell culture
Earlier, cell culture was extremely difficult, and three developments of the last 15 years have contributed to making it much easier: (a), by far the most important, the advent of non-toxic antibiotics to prevent microbial contamination; (b) standardisation and ready commercial availability of synthetic medium components such as a wide range of vitamins and amino-acids; and (c) availability of quantitative methods for evaluating cell growth, i.e., free-living, single-cell suspensions which are reproducible, easily sampled in a representative fashion and accurately counted. These factors allowed the systematic investigation of culture
conditions, and many techniques and fully or semi-synthetic media are now available for a variety of tissues.

For general techniques reference is made to a recent text-book of cell culture \(1\), but it will be useful here to summarise the differences of animal cell culture from standard microbiological technique, since advances in large-scale cell culture all stem from realisation of and allowing for these differences. They centre around the characteristics of the cells, which compared with bacteria are (a) much larger and heavier, although of similar density (and hence require more diligent agitation), (b) very much more fragile mechanically, and thus more readily damaged by agitation procedures, (c) much more susceptible to adverse chemical environment, (d) much more slow in growth (doubling time 12-48 h.). Consequently care has to be taken in their handling—they are damaged by grinding, too rapid stirring, sparging, frothing, certain but not all antifoam agents \(2\), too rapid centrifuging, keeping as a packed pellet, and incubation in acid (pH 6-5), alkaline (pH 8-0) or deficient media. Pumping in bulk requires special pumps such as those used for pumping whole blood. Also unlike bacteria, the cells prefer to attach themselves to solid objects, and consequently tend to clump or adhere to the vessel wall. However, on the credit side they are sedimented by low centrifugal forces and require a lower rate of oxygen input and acid removal.

Culture medium

The medium is also more complex and critical than those required for micro-organisms, and much more costly. This latter factor has been justifiable economically hitherto, because the products (virus vaccines) were relatively rare and are used in small doses, but more efficient vaccines demand higher doses; fortunately considerable scope exists for cheaper substitutes, for example use of skim milk in place of serum, and tryptic meat broth \(3\) in place of amino-acids or purified hydrolysates. Continuous cell lines may be developed which are independent of added protein and otherwise nutritionally less demanding. With adequate control, the expensive but very useful antibiotics might be omitted or used in lower dose. The medium needs to be isotonic with the correct balance of salts \(4\), with about thirteen amino-acids \(5\) (more than the seven required \(in\) \(vivo\)) and about seven vitamins \(6\) plus protein. The pH needs to be about 7-0, and the oxygen tension may be critical \(7\). There is now a very extensive literature indeed on tissue culture methods in general and in particular.

Cell source and methods of culture

There are three sources of cells for use in tissue culture, all suitable for large-scale application. The first and that entirely used
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Commercially so far is the intact animal, either from a part thereof (e.g., kidney) or the whole animal, usually an embryo (e.g., chick). Embryo cells are more easy to cultivate than adult tissue, and are frequently more sensitive to viruses. The organ is removed and coarsely chopped or minced; it can be used as this crude suspension of cell clumps (the first means used to make polio-virus vaccine), but better results are obtained by digesting the tissue with trypsin to obtain a single-cell suspension which can be maintained, usually as a single layer attached to a glass surface (monolayer) or as a suspension. Primary cultures do not increase greatly; they may be subcultured once or twice (giving secondary or tertiary cultures) but then usually expire. Primary cultures have the advantage of being less liable to loss by bacterial contamination, but suffer the disadvantage of frequently being contaminated with wild viruses.

The second source is that of the continuous cell lines, which have all resulted from infrequent successes in attempting to subculture primary cultures indefinitely; these successes occur sufficiently often to make it feasible to obtain almost any primary culture in a form capable of indefinite subculture, although certain types of cell are more difficult (8), and the resultant cultures may not necessarily have the same characteristics as the primary source. They are grown either as monolayers or in suspension.

The third source is the peritoneal exudate of certain ascitic tumors (9) which, while not strictly tissue cultures, nevertheless yield cells directly in a suspension eminently suitable for culture work. This source is in fact so bountiful (e.g., 1-2 x 10⁹ cells per mouse in 10 days) that it is surprising that more use is not made of it.

Principles of large-scale cell culture

Large-scale tissue culture of indefinitely cultivable cell lines is at present at a stage similar to that of penicillin fermentation at the time when deep culture was first introduced. The analogy is fairly close; the bulk of current commercial tissue culture is at present performed with the cells growing as monolayers, which are handled in separate flasks, as were the penicillin surface cultures. However, the same cells will in general also grow well in a shaken suspension if the right conditions are employed (10), and the system has been reduced to the simplified 'stirred pot' by McLimans and co-workers (11). This has been employed experimentally on a variety of scales up to several litres (12, 13) in batchwise growth and commercial exploitation is being investigated. Batch cultures are not efficient, however, since the optimal conditions for growth of animal cells are critical and are soon exceeded in culture; it would therefore be preferable to maintain the cells in a steady state under optimal conditions, with an apparatus analogous to a chemostat. Such an


**MICROBIOLOGY OF THE SOIL** (Margaret E. Brown, Ph.D., and R. Cooper, D.Phil.)

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This review deals mainly with nitrogen transformations by soil organisms. After years of slow progress, knowledge of the mechanisms of nitrogen fixation and nitrification is now increasing rapidly with the application of a greater variety of biochemical techniques; prospects for further advances are promising. Many nitrogen-fixing micro-organisms have now been described and their physiology and ecology studied. The complex relationship between plant and organism in the rhizosphere has been further unravelled and there are signs of reviving interest in the related subject of bacterial fertilisers, long neglected outside the Soviet Union. The organisms which attack insoluble phosphates in the soil and thereby increase the phosphate supply for plants have received more attention, and the recent findings are reviewed.

**Biological nitrogen fixation**

Recent work suggests that a great range of organisms fix nitrogen. *Derxia gummosa* was as efficient as *Azotobacter*, fixing 9–21 mg. N/g. of carbon source supplied, but was more acid tolerant than *Azotobacter* (1). Others, which were less efficient and fixed amounts of nitrogen ranging from 1 to 6 mg./g. of carbon source supplied, were identified as *Achromobacter* spp. (2), *Desulphovibrio desulphuricans* (3) and a possible *Saccharomyces* (4). Some species of actinomycetes may fix nitrogen but active forms were rare (5).
A CHEMICAL BASIS FOR THE CLASSIFICATION OF ANIMAL VIRUSES

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Besides satisfying the intellectually tidy, an efficient taxonomy is useful and necessary because it emphasizes resemblances which make worthwhile generalizations apparent, and makes minor differences easier to understand. The possible objection that viruses may not form a single natural taxonomic group need not inhibit attempts to define one. Several systems have in fact been proposed to classify animal viruses1,2 but have not proved very suitable, and virologists have generally agreed on the need for more data.

However, the earlier systems were based mainly on habitat at the level of the intact animal (that is, host-range, type of tissue usually affected, host-response and type of lesion produced, or vectors), and this now seems a major fault, since many objections can be levelled at such a primary subdivision3. For example, ‘obviously’ different viruses (foot-and-mouth disease and cow-pox viruses) can produce somewhat similar lesions, albeit at opposite extremities, of the same animal. Host-animal reactions may vary with the one virus (for example, chickenpox and herpes zoster4), and are a very complex expression of growth at the cellular level. Viruses can often be adapted to unusual hosts without much difficulty. Grouping by known hosts seems an arbitrary procedure, since many hosts may be unknown and thus only a few of the creatures potentially infected are selected; again ‘obviously’ different viruses (for example, myxoma and yellow fever viruses) can both depend on a similar vector (mosquitoes). Tissue tropism can be genetically very unstable; poliovirus can mutate in one step from neurotropic-plus-viscero-tropic, to purely viscerotropic, and back again5, and host-range variants of encephalomyocarditis virus are easy to obtain by a single growth cycle6. Cultured cells normally resistant to intact poliovirus can nevertheless support maturation of virus if infected with ribonucleic acid extracts of infected
cells. Adenoviruses have been found in stools, and might therefore legitimately be called 'enteroviruses', along with poliovirus.

It is therefore very doubtful whether habitat, as defined above, is at all suitable as a primary criterion for classification of animal viruses, although, as discussed below, it may be useful at another level of emphasis.

All systems of biological classification depend on mutationally stable criteria; indeed, the closeness of all biological relationships might in time be judged by the number of mutations which separate them, although this reasoning is easier to accept for the present when applied to sub-species and to interrelated micro-organisms. The difficulty with viruses is that they are genetically unstable when judged by human time-scales, appearing in a state of rapid evolution, so that a classification denoting species and genera is difficult to apply; attempts at a quantitative appraisal of the genetic differences without emphasizing one criterion more than another are likely at present to run into difficulties for lack of data.

What is needed to provide a lasting system for the classification of viruses is to obtain some primary and secondary sub-divisions which are not too arbitrary and which ought to be genetically quite stable. It is the purpose of this article to emphasize the need for genetic stability, and to point out the utility of a formal system based on lines of thought already familiar to many virologists, the high genetic stability of which, however, is possibly not fully appreciated as a classification criterion.

**Nucleic Acid as Primary Criterion**

The basis of the system proposed is the chemical structure of the 'virion', that is, the largest free-existing infective unit which can be defined as a single virus particle (usually the infective unit occurring naturally in largest numbers). It is primarily concerned with the type of nucleic acid contained, since this is the one component always essential for the production of infective virus; the chance of interconversion of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) by simple mutation is vanishingly small, if not impossible, so that this criterion should be quite stable. Another advantage is that this criterion must apply to all viruses, yet in an exclusive way. Present genetic concepts demand that a virus must contain either RNA or DNA or conceivably both; if the psittacosis group is excluded,
the existence of both in any virus is doubtful, but
the possible presence of RNA in a DNA-containing
virus (for example, the pox group) need not affect
the classification proposed. The only ambiguity
may arise in the hypothetical case where the same
total of information can be carried by either DNA
or RNA in a single form acceptable to a host cell,
thus giving two phases for the same virus. Fortu­
nately consideration of this objection can be deferred
until it can be raised experimentally. It is submitted
that the terms 'RNA virus' and 'DNA virus' are
terminologically improper; to avoid the clumsy
phrases 'ribonucleic acid-containing virus' and 'deoxy­
ribonucleic acid-containing virus', the terms 'ribo­
virus' and 'deoxyvirus' are suggested.

Essential Lipid as Secondary Criterion

The secondary sub-division uses the criterion of
ether sensitivity, suggested for classification purposes
by Andrewes and Horstmann. Destruction of
infectivity by lipid solvents (which appears to be
correlated with sensitivity to low concentrations of
detergents, and in the case of Newcastle disease
virus with sensitivity to lipase) presumably reflects
the presence of fatty components essential for infectiv­
ity. Those viruses insensitive to ether and detergents
either do not contain lipid (for example, poliovirus),
or their lipid content is tightly bound or not essential
(for example, vaccinia). The genetic stability of
this criterion is less certain than that of difference in
nucleic acid content, but two facts suggest that it is
non-trivial and that it should be fairly stable (that is,
not readily lost by non-lethal mutation): (a) the
ether-sensitive particle of influenza virus breaks down
completely when the lipid is removed; (b) among
riboviruses, the sensitivity to ether correlates with
maturation at the cell wall, suggesting a widespread
mechanism involving lipid materials without which
the viruses cannot become infective.

In practice and excepting the pox group, viruses
appear either to be quite stable to ether, or to be
very sensitive (provided that they are tested in an
accessible state and not protected within a mass of
tissue), making it a fairly easy criterion to apply.
Those ether-resistant viruses so far examined (for
example, poliovirus, adenovirus, polyoma virus)
have a strong and regular lattice-like (clathrate) sub­
structure, whereas the ether-sensitive viruses are less
rigid, tending also to flatten more in electron micro­
scopy. Accordingly, the terms 'clathrovirus' and
'lipovirus' are suggested for the ether-resistants and
ether-sensitives respectively, to apply to the infective form only (as some lipoviruses, for example, herpes virus, may contain clathrate substructures).

Consideration of Major Groups

When these two types of separation are made, and the viruses the positions of which are known then arranged in each of the four major subdivisions in order of size, a considerable degree of order results (Table 1), and the viruses are brought together in groups which are often immunologically related. Four principal chemical relationships are therefore used: nucleic acid type, content of essential lipid, size and immunological character; it will be noted that 'chemical' is here used in its widest sense, and that among entities of the size of viruses there is little difference between chemical content and fine morphology. There will, of course, be gaps, many known viruses cannot yet be placed, and the placing of some viruses (marked with a query) is based on rather slender data, but the position of most is reasonably well based for at least one member of a serological group.

It will be noted that the pox-viruses are treated as a special case, and this is for two reasons. First, separation into ether-sensitive and ether-resistant has the effect of separating myxoma/fibroma from the vaccinia and avir-pox groups, and, although there are some reservations about their relationships, recent work shows their closeness by reactivation and serological data. Secondly, vaccinia nucleic acid clearly replicates in the cytoplasm, whereas the nucleic acid of all other viruses adequately studied (herpes virus, adenovirus, encephalomyocarditis and fowl plague) replicates in the nucleus. The pox viruses therefore seem metabolically more autonomous than smaller viruses, and there is a strong case for regarding them as a distinct group intermediate between the smaller viruses and the ornithosis group.

Because of its inherent genetic stability the nucleic acid type is preferred as a primary criterion, and, because of its observed genetic instability in some cases, immunological character is set last, but the relative stability of the other characters is not predictable. The viruses also come together in groups of similar habitat, and it may be that this admittedly useful grouping, while unsuitable as a primary criterion, could be applied as a tertiary one. Deoxyviruses are generally larger than riboviruses although overlap does occur. Lipo-riboviruses tend
to be larger than clathro-riboviruses. Thus some generalizations can be made from this system, since the primary and secondary criteria also separate other properties, and this should help the understanding of the structure and growth mechanisms of new viruses if these criteria can be applied.

Conversely, other properties can help classification when the criteria cannot yet be applied. For example, the large size of measles virus, the formation of large intranuclear inclusions and its intracellular mode of growth all suggest that it is a deoxyvirus. Rous sarcoma and vesicular stomatitis viruses are both lipoviruses; there is evidence, not firmly based, that both are riboviruses, and their continuous mode of release strongly supports this idea.

At the moment there appears little evidence to contra-indicate the classification of Table 1, although other arrangements are possible; for example, one may regard size as more important than ether sensitivity and promote it to secondary criterion, but the large differences in size among the smaller viruses makes deciding the borderline arbitrary and inconvenient. From a practical point of view, determination of reaction to ether is much easier than determination of size. Good evidence against the proposals would be the finding of strong serological relationships between the main groups differentiated in Table 1.

Correlations with Growth Properties

The separation into four main groups accords well with growth properties, where these are known. In fundamental replication mechanism (that of the nucleic acid) one must expect deoxyviruses to resemble one another more than riboviruses, and vice versa, although a possible exception to this is the small single-stranded DNA-containing phage, the replication of which may resemble a ribovirus. The deoxyviruses (vaccinia, herpes and adenovirus as examples) are slow to mature and to be released, and form marked inclusion bodies, but the riboviruses mature much faster and do not form marked inclusions.

The lipo-riboviruses (for example, vesicular stomatitis, influenza, western equine encephalomyelitis, fowl plague) appear to be matured at the cell wall, whereas the clathro-riboviruses mature internally. (for example, poliovirus, encephalomyocarditis, foot-and-mouth disease, vesicular exanthema), probably in the cytoplasm. This differentiation does not apply.
Table 1. A Classification Scheme for Animal Viruses Based on Nucleic Acid Content, Sensitivity to Ether or Detergents, Size and Serological Relationships

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Classifications</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POX-VIRUS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEOXYVIRUS (DNA-containing)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smaller deoxyvirus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clathrovirus (other sensitive)</td>
<td>Myxoma Fibroma Ether-resistant Animal pox (vaccinia, etc.) Avian pox (fowlpox, etc.)</td>
<td>200-300</td>
</tr>
<tr>
<td></td>
<td>Adenovirus *</td>
<td>70-90</td>
</tr>
<tr>
<td></td>
<td>Clathrovirus (other resistant) Rabbit papilloma</td>
<td>40-50</td>
</tr>
<tr>
<td></td>
<td>SE polyoma</td>
<td>40-50</td>
</tr>
<tr>
<td>RIBOVIRUS (RNA-containing)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular exanthema, Reovirus (ECHO 10)?</td>
<td>Poliovirus* ECHO 8 viruses Coxsackie A7, A9, B1, B4, B5</td>
<td>50-70</td>
</tr>
<tr>
<td></td>
<td>Certain enteroviruses - EMC, CoxsE, MM, Mengo*</td>
<td>27-32</td>
</tr>
<tr>
<td></td>
<td>Foot-and-mouth disease</td>
<td>22-25</td>
</tr>
</tbody>
</table>

* Serological group. Data on vesicular exanthema from Shaffer and McClain (personal communication)

It is interesting that viruses capable of causing proliferative lesions, even if finally necrotic, occur in all groups save the clathro-riboviruses; hemagglutinins of one sort or another are also found in all groups except the lipo-deoxyviruses, indicating that neither of these phenomena is related to a particular
virus type, and is therefore per se of small value for classification purposes.

Means of including more Viruses

It may be felt that it is impractical to determine the nucleic acid present in a large number of viruses in order to classify them, even if one selects only a single member from each serological group. However, this information is urgently necessary, since it is an essential preliminary to understanding the most fundamental part of their growth processes, representing probably the most important single datum, and providing a most useful guide to selecting models for study.

The most direct way of determining the nucleic acid is to examine the purified particle, but this is probably also the most lengthy, as some viruses, particularly the small thermolabile ones, are difficult to purify. Fortunately, there is at least one apparently conclusive and fairly direct short cut. This is to obtain the nucleic acid in an infective form susceptible to nucleases (usually as the free acid), and then to determine its sensitivity to the two species of nuclease. This has now been done with poliovirus, encephalomyocarditis, mengo, Eastern equine encephalomyelitis, Semliki forest, West Nile, Murray Valley encephalitis and foot-and-mouth disease viruses among the riboviruses, and polyoma among the deoxyviruses, and also for the DNA-containing bacteriophages T2 (ref. 37) and X174 (ref. 38); some viruses have so far proved resistant to successful extraction, but may yield to improved techniques.

A more readily applied, if less direct, method is to test the growth-inhibitory effect of 5-fluorodeoxyuridine, which should specifically inhibit DNA synthesis. This compound inhibits growth of a deoxyvirus (vaccinia) but not of a ribovirus (poliovirus), and gives promise of reliable results provided that it can be confirmed with more known examples and that it is used with a host cell which it penetrates successfully (that is, in which it inhibits a known deoxyvirus). This is a very simple test, and there is a strong case for all virus laboratories to run it through their collection of virus strains, as the information to be obtained is of primary importance. A preliminary screening of new clinical isolates, for example, with 20 per cent ether and 5-fluorodeoxyuridine, might speed typing and economize in antisera.

Classification of all Viruses

It is tempting to follow this scheme to its conclusion and include all viruses in the one classification,
since superficially plant and bacterial viruses seem separated from animal viruses only by host range. This would have the effect, satisfactory from a structural point of view, of placing all bacteriophages and Tipula iridescent virus alongside the adenoviruses, and plant viruses (for example, tomato bushy stunt virus) with poliovirus; the embarrassment of deciding the correct 'kingdom' for plant viruses which grow in insects (and vice versa) would be avoided. In fact, the fundamental likeness in all biological replications and cellular metabolism makes it nowadays legitimate to question seriously whether the usual separation into plant, animal and bacterial viruses is justified, because this classification may depend only on relatively trivial differences in the virus coating. Convincing evidence of this would be growth of a virus in a host of the 'wrong' kingdom; this might be achieved by unusual manipulative techniques, provided that there is no fundamental impediment. Alternatively, evidence for such an impediment would itself constitute most important information.

Conclusions

In summary, a taxonomy for animal viruses based on type of nucleic acid and essential labile lipid content appears less arbitrary and genetically more stable than those previously suggested, and may provide a useful means of ordering the host of viruses now newly coming to light. The typing of the nucleic acid, a factor of primary importance, is likely to be easier than was hitherto supposed, requiring not very accurate assay procedures but needing some system in which infectivity can be detected; it merits wide consideration. Such a taxonomy might in time include all viruses.

THE HAZARDS INVOLVED IN LARGE-SCALE HANDLING OF POLIOVIRUS*

P. D. Cooper

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Within the last five years or so a phenomenon very familiar to science has been repeated yet again, this time in the virus field. A rare laboratory curiosity has become a commonplace and a household word, and poliomyelitis vaccine will nowadays be a curiosity to few, least of all perhaps the school children. The reason for this is that technical developments have made it much easier to grow this hitherto difficult virus in culture. Some of these are in the nature of a "break-through" exemplified by Enders' Nobel prize-winning discovery that, although polio grows destructively in the nervous system of animals, nervous tissue is not necessary for culture of the virus outside the body. The kidney of the monkey, which is much easier to grow, can be substituted. Other developments are the steady improvements in standardization of medium components, and particularly the improvements in handling cells in culture so that cells and viruses can be sampled reproducibly and counted accurately; the best conditions for growing them can then be worked out systematically, thoroughly and quickly.

Having these facilities it only required the right incentive to be able to grow many viruses on a very large scale, and of course the incentive was provided by the need to produce an effective vaccine against poliomyelitis. Large-scale production of this vaccine was started in the United States in 1954-5, and extended a year or two later to this country; it is no exaggeration to say that enough poliovirus is produced now every minute throughout the world, to infect every human inhabitant of the world.

However (and this qualification will apply to all virus vaccines), the need to provide a vaccine implies that the infectious agent carries a considerable hazard of its own. Therefore production of any worthwhile human vaccine will contain some element of danger, particularly in the early research and development stages, and it is these hazards that will be discussed now.

Appraisal of the hazards presented by poliovirus

Poliomyelitis (the newly approved name of the virus of poliomyelitis) is no exception to this rule, although the hazards are not so

severe as they may at first appear. In fact, of the score or so people throughout the world who have died as a result of their work on poliovirus, nearly if not absolutely all were infected, not with poliovirus, but with much more dangerous wild viruses from the monkeys which were being used as a source of tissue. This is an ironic result, and perhaps represents some small operation of the law of averages, bearing in mind the hundreds of thousands of monkeys which have been sacrificed.

Monkeys themselves therefore represent a hazard, but this is dealt with elsewhere in this symposium, and what will be considered here are those hazards which will be present in or around an infected animal house using virus on a very large scale, but peculiar to the virus itself. It should be emphasized that it is much easier to handle a pathogen in a test tube with a cotton-wool plug and a wire loop, than it is to cope with it in a 20 litre fermentor; above this scale, which comprises a container rather too heavy for one person to lift with safety, the hazards do not change greatly with a further increase in scale. Thus, hazards depend greatly on the size of the operation.

Before considering poliovirus itself any further, however, it is necessary to indulge in a little philosophy because, in handling poliovirus, a certain compromise has to be made.

It is this: for many purposes, a pathogen must be regarded either as dangerous, or not dangerous. If it is not dangerous, no further consideration is needed here, but if it is dangerous, then it must be treated in complete isolation. By this is meant the precautions used for very radioactive sources, namely, remote control handling, isolation in sealed vessels, and careful checking of the health of personnel. This may seem care over-exaggerated, but in fact these methods are used for certain pathogens, particularly in experiments or preparations in which aerosols are produced; any departure from this would involve serious hazards for the operators. Needless to say, however, such procedures would greatly slow the rate of working, and would add enormously to the cost of equipment needed.

In the light of these thoughts, has poliovirus been generally regarded as dangerous or not? The answer lies in the compromise mentioned above; that it contains a hazard is well recognized, but the hazard is of a low order. Three groups of virus pathogens may therefore be recognized from a hazard view-point:

1. "Open" viruses, i.e. those normally administered alive to humans, such as vaccinia and attenuated yellow fever; here the main pre-occupation is to keep contaminants out rather than virus in.
2. "Closed" viruses—variola being a good example of a very virulent virus.

3. "Partly restricted" viruses—for example, poliovirus.

   The reason for the low esteem in which the virulence of poliovirus is held resides largely in its widespread occurrence in normal "healthy" populations, in contrast for example to smallpox. It is a natural hazard; in a non-vaccinated population a very high proportion, if not all, have contracted the disease subclinically by adulthood (as evidenced by antibody production), and have shown no ill effects. The proportion permanently affected by poliomyelitis is relatively very small, and is mainly brought to public notice by a low mortality (in contrast again to smallpox), in which most of the victims remain at large to demonstrate their clear symptoms which involve the onlooker emotionally. It is fortunate for virology, in a way, that poliomyelitis presents to some extent this emotional problem, since this has acted as a spur to produce the methods which should in time be available to counter other less well-publicised infections.

   Thus the chance of a person being susceptible to poliovirus is small, and by selecting immune individuals in the first place, and later vaccinating them, risk to personnel is very greatly minimized.

Hazards affecting the work

   Most of the precautions needed to protect the work are also those needed to protect the workers, and will not be considered separately. The main precaution necessary for the work alone is to avoid contamination with the ordinary laboratory contaminants (bacteria, moulds and yeasts), and to avoid mixing the strains of virus, and this last is encompassed in vaccine production preferably by using entirely separate buildings for each strain, or by using laboratory space, perhaps made of stainless steel, which can be steam-disinfected.

Hazards affecting the workers

   It is possible to contract poliomyelitis by a variety of routes, all of which will be met in handling poliovirus both in the animal house and in the laboratory. The infected monkey will represent a source of virus, although often the virus is administered by intracerebral inoculation, and this will kill or paralyse the animal without liberating much virus to the environment. The normal mode of spread in human populations is probably largely by direct contact of faecal contamination on hands and exchangeable articles, with a small
contribution by hand and droplet infection from nasopharyngeal material. Virus is only found in the throat for a few days, but remains in the gut for many weeks, and large scale safety testing of live virus vaccines may involve very large quantities of contaminated excreta with the possibility of some virus more virulent than that fed to the animal. Spread from monkeys can be expected to be similar to spread from humans, with dust and wet contaminants from bedding and the remote chance of an infected bite in addition. Spread from non-primates, in which poliovirus does not grow at all significantly, is a negligible hazard.

In the laboratory, the biggest chance of infection is probably by direct ingestion of liquid from, say, contaminated fingers, cigarettes or pipes, or clothing. From aerosols, if freshly formed say by excessive foaming, infection is a possibility but the droplets soon dry in air and in general desiccation greatly reduces the infectivity of poliovirus. That which does survive, however, is rather stable, and in liquid media the virus will survive for many months, or years below 10°C.

Methods of protecting personnel

As mentioned above complete isolation of the virus is desirable, but in practice the compromise is taken with poliovirus of only partial isolation, for reasons of cost and speed of working.

The main precaution is that of containing virus within a strictly limited area, from which no virus can leave except in sealed and non-breakable containers or through an autoclave adequate for sterilization. For this, good pre-planning and special building is essential. Virus is preferably manipulated in separate cubicles which can be disinfected easily. Nearly all viruses are fortunately very sensitive to thermal inactivation. It is important to prevent virus from entering the drainage system. Virus-containing fluids are transferred by gravity flow or by pumps which do not liberate aerosols, and any spillages are mopped up with disinfectants (sodium hypochlorite being probably the most effective). Outer protective clothing reserved for “infected” work is worn, and facilities should be available for rapid showering and changing should any personal clothing be contaminated. Absorbent cotton wool plugs, while not sufficient to hold up virus, are adequate for containment of aerosols, and face-masks are sometimes worn for the same purpose. Ducted air effluents may also be sterilized by heat or UV lighting; this is in theory 90-99% effective, but the amount of live virus leaving in unsterilized air, even if an aerosol has occurred, is probably very small, unless the dust content is high.
Staff must of course be trained in the employment of ordinary good sterile technique, that is aseptic working, avoidance of aerosols, liberal use of disinfectants and UV light; however, some procedures, notably use of the Spinco ultracentrifuge and Seitz filtration, are notoriously difficult to use cleanly. For such work rubber gloves, aprons, boots and face masks should be used. Personal hygiene must be adequate, since poliomyelitis is a “social” disease. A great advantage with poliovirus is that all staff can now be vaccinated and tested for efficacy of the take, which probably gives better than 90% protection. A sound course is also to vaccinate all primary contacts, such as the immediate family and co-workers of staff, and if possible to vaccinate secondary contacts as well, such as colleagues in virus-free departments, so that if virus is carried on or inside a person, the chance of an epidemic being started is blocked at the outset.

It should be emphasized that the risks entailed with poliovirus in the usual operations in the animal house are considerably less than those met with on the much larger scale somewhat artificially produced in the laboratory, and in any case much less than the natural hazards of handling monkeys. In conclusion one can say that the hazards of handling large amounts of poliovirus are not intrinsically very great, and are nowadays reduced by the application of the fairly simple procedures described to relatively negligible proportions.

Needless to say, the smaller the scale of working the easier these precautions become, and considerable improvements can be made by decreasing the bulk handled (whether of liquid volume or of number of monkeys) while keeping the amount of virus the same or greater—in other words increasing the efficiency of operation. There is a good chance that the next ten years will see improvements of this nature.

DISCUSSION

Dr. C. R. Coid: Would Dr. Cooper agree that animals injected with poliovirus strains do not present a real hazard to technicians who (a) have a reasonable level of antibody (b) wear suitable protective clothing?

Dr. P. D. Cooper: It is clearly not possible to give a definitive answer, since this will depend upon the virulence of strains handled, the route of administration of the virus to monkeys, the scale employed, the facilities available and the routine care with which these facilities are used. However, routine parenteral safety testing of inactivated vaccines will present a quite negligible risk, and in the testing of live virus vaccines the risk will not in practice be much greater, certainly less than the risk of handling “uninfected” monkeys.
Plaque assays were introduced for animal viruses by Dulbecco (1952) as a direct analogy with the plaque assay of bacteriophage (Ellis and Delbrück, 1939), with the object of improving the methods of handling a wide range of animal viruses so as to obtain more rigorous information. One aim was to count individual infective particles in the same way that
individual bacteria or bacteriophages can be counted, to assay virus infectivity more accurately, and to express virus concentrations in terms of these particles rather than as arbitrary units; another was to achieve the simplification that tissue culture brings compared with animals or fertile eggs, and a third was unequivocally to isolate clones of virus from single infective particles, thus facilitating genetic studies.

This very useful and original adaptation was made possible by preceding developments in tissue culture, in particular the development of relatively simple and reproducible media containing antibiotics, and methods for preparation of monodisperse free-living and virus-sensitive cell suspensions, either as continuously cultivated cell strains, or from intact animals. Organs which were apparently insusceptible to the virus in vivo were often used.

Dulbecco's method with Western equine encephalomyelitis virus and chick embryo cells was foreshadowed by the observation of microfoci of Eastern equine encephalomyelitis virus growth in cell sheets of rat sarcoma cells by Gey and Bang (1951), who briefly suggested it as a means for titrating virus units. In the nine years or so since its first use it has seen extensive and increasing application to different systems; there are now more than two hundred reports on work relating to or using the plaque assay, and it has clearly become a method of some importance. However, it has by no means completely replaced earlier methods, such as 50% infectious dose and pock count assays, and it is unlikely to do so; nevertheless as new viruses become established by means of older methods, increasing demands for accuracy and economy of working coupled with increasing familiarity with tissue culture systems have encouraged the tendency to replace these methods with plaque assays.

This article will not be concerned with historical development, however; its purpose is rather to consider in detail and from a practical viewpoint the many factors which contribute to the accuracy and convenience of the plaque assay, in order to reach some understanding of them, to improve existing methods and to assist their application to novel systems. "Plaque assays" will be defined as methods enabling counting of local lesions in tissue culture; the principles and methods of estimation of virus particles have been dealt with by Isaacs (1957), who discussed the plaque method briefly.

II. Glossary of Abbreviations Used

VSV       Vesicular stomatitis virus
NDV       Newcastle disease virus
FMD       Foot-and-mouth disease
WEE       Western equine encephalomyelitis
THE PLAQUE ASSAY OF ANIMAL VIRUSES

EEE  Eastern equine encephalomyelitis
EMC  Encephalomyocarditis
CPE  Cytopathic effect
RNA  Ribonucleic acid
DNA  Deoxyribonucleic acid
PFU  Plaque-forming unit
ID_{50}, TCD_{50}  50% Infectious dose, 50% tissue culture (infectious) dose,
LD_{50}  50% lethal dose

III. GENERAL CONSIDERATION OF THE PLAQUE ASSAY METHOD

A. The Principle of the Method

In outline, the plaque assay consists of the inoculation of a statistically adequate yet easily countable number (say 30–100) of infective virus particles onto an immobilized layer of cells. Each particle is allowed to replicate under conditions where the resulting lesion remains local, and the lesions are finally counted. It can be shown that each lesion is caused by a single particle, and this enables the calculation of the infective particle content of the original inoculum.

B. Differences from Other Methods

The infectivity of virus preparations can be measured either in abstract units which do not allow direct interpretation (e.g., in terms of "survival time"), or in terms of real units, such as the individual infective particles. Real units are clearly preferable for their ease of visualization, and two methods of counting them exist. One is the end-point method, giving an all-or-none response and in which virus is diluted until only a few inocula contain a single infective particle, and the other is the lesion-counting method, in which inocula are all diluted to contain about 100 particles; on inoculation into a substrate containing living cells one either scores for growth (positive or negative) or counts individual lesions, respectively.

The plaque assay method is distinguished by one or both of two differences from all other methods; (a) being a lesion-counting method it produces many lesions in one container (a tissue culture) rather than occupying a whole container with one lesion or wasting it by a total absence of lesion; (b) it uses a very reproducible substrate, namely tissue culture. The increased accuracy of the plaque assay is achieved partly by use of such cultures and partly by its economy, since equivalent accuracy could be obtained by the end-point method by use of a larger number of containers.
Provided that one is concerned simply with accurate comparisons of infectivity, there are several reasons for preferring tissue cultures to *in vivo* or *in ovo* systems: 

(a) variations between individual cultures of a batch, and between batches made at different times, can be made quite small, 

(b) the number of cell types present can be reduced to one, 

(c) humoral reactions and other variable factors affecting sensitivity can be eliminated, 

(d) virus inactivation can be controlled, 

(e) one can be sure of dealing with a single particle, 

(f) in studies of virus neutralization, excess antiserum can be removed by washing after adsorption of surviving infectious units. These advantages apply equally to end-point titrations in tissue culture and it is not the present purpose to discuss tissue culture-virus systems in general; the essential difference between plaque assays and other tissue culture methods is the localization of virus infections by means other than confinement in individual containers, so greatly increasing economy of time and materials.

In principle this localization is identical with the localization of bacteriophage plaques or bacterial colonies, in which free diffusion of the infecting organism is slowed or prevented by a gelled substrate (usually agar medium); spread by convection or mechanical currents is completely prevented. Occasional viruses are self-limiting in spread and require no artificial localization. In all cases the prime requirement is a discrete form of virus growth which can be distinguished macro- or microscopically from a background of uninfected cells; the function of the cells is therefore both to support growth of the virus and to provide a contrasting background against which virus growth can be recognized.

**C. Mode of Development of a Local Lesion**

Having achieved localized changes due to virus, there appear to be three ways in which such changes may spread and thus become recognizable. By far the commonest is by release of free virus into the medium, which then spreads by simple diffusion to reach and infect adjacent healthy cells; in this case antiserum in the medium prevents development of a plaque. Dulbecco and Vogt (1954a) have found viable poliovirus 3–4 mm. beyond the visible limits of a plaque. While it seems that all viruses can spread by this means, in a few cases diffusion probably contributes little to plaque or focus development. Herpes B virus is of a size to diffuse poorly through agar (Polson, 1956), yet, even in the presence of antiserum, foci continue to enlarge, apparently by syncytial formation (Black and Melnick, 1955); cells fuse and thereby become bigger and eventually disintegrate, the infection being transmitted by direct cell-to-cell contact. This mechanism is generally limited to the larger viruses. With Rous sarcoma virus (Temin and Rubin, 1958) on the other
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hand, cells are not destroyed and only very small amounts of virus are released; in this case the focus enlarges mainly by growth and division of the newly created Rous sarcoma cell.

D. Criteria and Requirements for a Reproducible Plaque Assay

Most of the factors affecting the assay will be discussed in detail below, but it may be useful to list certain criteria for their adequate control at this point.

In order to demonstrate the usefulness of a plaque assay it is essential to show the following points, the importance of some being self-evident.

1. There must be no "plaques" in absence of virus.

2. The characteristic virus must be regularly reisolable from a plaque in far higher concentration than from areas away from a plaque.

3. Plaques must follow a linear relationship with dose (and not with dilution), i.e., plaque count must be proportional to virus concentration, at least over a range within statistical practicability.

4. Plaques should be spread among cultures of one batch according to a Poisson distribution, and the coefficient of variation of duplicate assays should be near the value statistically expected from the total number of plaques counted.

5. Plaque initiation must be inhibited by low concentrations of heated specific antiserum which will not inhibit growth of another virus in the same cell system (to eliminate the possible presence of anti-cell antibodies), and not be inhibited by normal serum of the same source species.

6. Repeated assays of a preserved virus stock in cultures of different batches should give the same titer.

The most important criterion for assay purposes, (3), has been amply demonstrated for many systems and the remainder have been shown for some others, but it is rare to find all criteria employed. Additionally it is desirable to demonstrate that all cells have quantitatively equal sensitivity (cloning in the case of continuously cultured strains); the virus also should be cloned, be shown to be genetically stable, and stocks should be purified and subcultured in dilute passage, although these things have rarely been done.

E. Choice of Culture System

There are broadly two ways in which cells may be cultured while retaining a localization of virus growth, that is either (a) as a confluent sheet on a surface (monolayer) or (b) suspended in a layer of gelled substrate, usually agar (agar cell-suspension). The variables involved at
each stage of the methods will be discussed in detail in Section IV, but the general procedures can be outlined as follows.

1. Monolayer Plaque Assay

Cells are added in appropriate concentration, volume, and medium to a flat-bottomed container suitable for tissue culture (e.g., neutral glass petri dishes or capped bottles), and allowed to settle and attach (3-20 hours). Usually a period of growth, 6-14 days, is then required, particularly for epithelial cells, to fill the surface completely with cells. The medium is then removed and, with or without washing, suitable virus dilutions containing 20-200 PFU are added in small volume, and a predetermined period allowed for virus to absorb to the cells. After adsorption, liquid nutrient agar medium is added and allowed to set on a level surface, and the plates or bottles are incubated in a suitable temperature and atmosphere for a predetermined time (30 hours-21 days, but usually 2-4 days). For some viruses the agar can be omitted from the nutrient medium. Usually plaques can be seen by indirect light against a dark background, and occasionally are distinct enough to count in this way, but nearly always some stain, usually neutral red, is added to enhance the contrast. After a period for the cells to absorb the stain (usually 2-4 hours) plaques can be counted.

2. Agar Cell-Suspension Plaque Assay

This method is essentially similar to the monolayer method, except that the nutrient agar layer is added to the container before the cells and is allowed to set. These base layers can be stored quite well. A small tube containing an appropriate number of cells in a small volume of medium receives a dilution of virus similar to that added to monolayers, followed by an equal volume of liquid agar medium, the whole being rapidly mixed and poured at once on to a base layer and allowed to set. No adsorption period is necessary. Subsequent treatment is then identical to that of the monolayer method.

3. Comparison of Monolayer and Cell-Suspension Methods

The relative merits and demerits of these two systems are compared in Table I.

The monolayer method was the original introduction by Dulbecco (1952), and has been employed the most extensively. It is particularly useful for rare or expensive primary cultures, such as monkey kidney, which it is desired to expand as much as possible by growth before use, and then to use for plaque assays in the original growth containers. More attention is required, however, than if the cells are grown in bulk. If
### TABLE I

**A Comparison of the Monolayer and Agar Cell-Suspension Plaque Assay Methods**

<table>
<thead>
<tr>
<th>Monolayer method</th>
<th>Agar cell-suspension method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduced 1952 (Dulbecco)</td>
<td>Introduced 1955 (Cooper)</td>
</tr>
<tr>
<td>Sensitivity higher for herpes virus* and probably for pox group*</td>
<td>Sensitivity higher (up to 7×) for free virus and infected cells of: fowl orphanh, vesicular stomatitis, FMD, poliovirus*</td>
</tr>
<tr>
<td>Infected cell assays usually require additional maneuvers</td>
<td>Probably cannot be used for viruses &gt;120–150 μm diameter</td>
</tr>
<tr>
<td>Virus has long diffusion path in “spot tests”; stain diffuses slowly</td>
<td>*Particularly useful for infected cell assays</td>
</tr>
<tr>
<td>Prediffusion less easy in plaque inhibition tests</td>
<td>*Short diffusion path for “spot tests”; stain diffuses quickly</td>
</tr>
<tr>
<td>Depth of agar often critical</td>
<td>*Prediffusion gives high sensitivity in plaque inhibition tests</td>
</tr>
<tr>
<td>*Most useful where cells in short supply (e.g., primary cultures of small organs)</td>
<td>*Depth of agar not critical</td>
</tr>
<tr>
<td>—cells can be multiplied and used in same container</td>
<td>Requires more cells for primary cultures</td>
</tr>
<tr>
<td>Cells grown in small monolayers require more attention than cells grown in bulk</td>
<td>*Requires same or less cells for continuous strains (KB, ERK)</td>
</tr>
<tr>
<td>Cells can be “stored” as monolayers at 30°</td>
<td>*Cells can be grown in bulk; less chance of contamination</td>
</tr>
<tr>
<td>Cultures have to be prepared in advance, usually 1–6 days</td>
<td>Cells can be “stored” in suspension at 30°</td>
</tr>
<tr>
<td>Cell concentration is rather critical (for confluence)</td>
<td>*Cells can be used at any time at short notice, from monolayer or suspension cultures</td>
</tr>
<tr>
<td>Cells more anaerobic, make more acid</td>
<td>*5–10× range of cell concentration can be used</td>
</tr>
<tr>
<td>Can only be used for cells forming monolayers</td>
<td>Cells more aerobic</td>
</tr>
<tr>
<td>Mixed cell populations may form localized colonies or overgrow</td>
<td>*Can be used for all cells; essential method for ascites cells which do not form monolayers</td>
</tr>
<tr>
<td>*Nonviable cells can be removed by washing before plating</td>
<td>*Mixed cell populations are fixed and cells generally do not grow in agar</td>
</tr>
<tr>
<td>State of culture surface fairly critical</td>
<td>Cell viability must remain high</td>
</tr>
<tr>
<td>A 0.5 to 4-hour adsorption period required</td>
<td>*Independent of culture surface; glass cleaning is easier, and method can be used with cheap plastic dishes that will not grow monolayers</td>
</tr>
<tr>
<td>*Cells can be examined microscopically for CPE</td>
<td>*No adsorption period necessary</td>
</tr>
<tr>
<td>*If agar and killed cells are removed, fixative stain (e.g., crystal violet) can be used</td>
<td>CPE is generally not visible unless gross</td>
</tr>
<tr>
<td>Specific cell stain must be used, e.g., neutral red, tetrazolium, or trypan blue</td>
<td></td>
</tr>
</tbody>
</table>
TABLE I (Continued)

<table>
<thead>
<tr>
<th>Monolayer method</th>
<th>Agar cell-suspension method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaques sharply defined but small</td>
<td>Plaques larger but less sharply defined, especially at low cell concentration</td>
</tr>
<tr>
<td>*Plaques can be developed by “hemadsorption”</td>
<td>Presumably difficult to use with hemadsorption development</td>
</tr>
</tbody>
</table>

*Advantage lies with the system marked.

subcultured at any stage then the particular convenience of the monolayer assay is lost. The monolayer method is also more suitable for very large viruses which diffuse slowly through agar (e.g., herpes simplex, Waterson, 1958b), for assays which take more than a week to develop (this may be a question of improving the medium to preserve cells in agar suspension), for microscopic determination of foci, for plaque contrast enhancement by fixative strains, or for plaque development by hemadsorption, where cell-to-cell contact is required.

The agar cell-suspension method was developed by Cooper (1955) specifically to obtain a higher efficiency of plating of infected cell suspensions, and to approach the versatility of bacteriophage assays. It turned out to have a number of other advantages, mainly its higher efficiency of plating for free virus of the small- and medium-sized groups ($7 \times$, fowl orphan virus, Macpherson, 1960; 1.3–1.5$ \times$, VSV, Cooper, 1955; 1.6–2.8$ \times$, FMD virus, Sellers and Stewart, 1959) and its generally greater convenience of handling (Cooper, 1961a). Since cells are always obtained at harvest in suspension, it is convenient to keep them in this form; plates can be poured at short notice, no adsorption period is necessary, cell concentration, glassware and agar quantities are generally less critical, “spot” and plaque inhibition tests are higher in sensitivity, and it is easier to use mixed cell populations. The amount of cells required for some primary cultures appears to be higher than needed for monolayers, but the number of KB and ERK cells required is if anything slightly less than for monolayers; it is probable that the need for more primary cells could be met by improving the medium. There is no alternative to the agar cell-suspension method for ascites tumor cells (Sanders, 1957), which do not form monolayers on glass. Cell suspensions can also be used to “develop” plaques in a cell sheet prevented from absorbing stain by X-irradiation, (Franklin, 1958a).

Thus it can be seen that both methods have individual merits, depending mostly on the virus used and its growth characteristics, but also on the cells available and the manipulations involved. The choice de-
pends on the sensitivity required and the particular convenience and applicability, having regard to the selected cell-virus system and information needed. Apart from the exceptions noted in favor of the monolayer method, the weight of advantage would appear to be with the cell-suspension method, particularly for laboratories newly setting up plaque assay systems.

IV. TECHNICAL FACTORS AFFECTING THE PLAQUE ASSAY

Reproducible, accurate, and sensitive plaque assays depend upon the control of a number of experimental variables, and this section will discuss these factors in some detail.

A. Source of Cells and Preliminary Handling

It is not proposed to discuss extensively the general technique of handling cells for plaque assays, since this is common to all tissue culture, but rather to summarize its particular application; for media, glassware, and methods reference is made to a general textbook on cell culture techniques (e.g., Paul, 1959).

At some stage of the procedure, either monolayer or agar cell-suspension plaque assays require a suspension of cells, largely viable and not in large clumps. The requirement for monodispersion is variable, but may be summarized as not being particularly necessary for agar cell-suspension assays, but much more so for monolayers that will be used within a few hours, since the cells will have no time to arrange themselves more evenly. Usually monolayers are left for 1-6 days, during which time cells divide and migrate from clumps to form an even sheet, and the non-viable cells do not attach and can be washed free. Those cells which tend not to separate easily from clumps are inconvenient to use, since the clumps may wash off or necrose, simulating plaques.

Cell suspensions may be obtained from three sources: (1) directly as a primary culture from an intact animal, or one or two subcultures therefrom (secondary or tertiary cultures), (2) from cell lines continuously cultivated in vitro, (3) from cell lines continuously cultured in vivo in ascitic form (solid tumors do not differ in practice from source 1). Source (3) has characters in common with both (1) and (2).

1. Directly from Intact Animals

Primary or secondary cultures have usually been made from a restricted variety of sources, the most common being kidneys from monkeys or other mammals (giving largely epithelial cultures), and the cells from the entire 8- to 12-day-old chick embryo, minus head, viscera, and limbs; both sources were originally described by Dulbecco (1952) and
Dulbecco and Vogt (1954a). Useful modifications are those of Bodian (1956), Rappaport (1956), and Bishop et al. (1960) for monkey kidney, which no doubt can be applied in principle to other systems. In practically all procedures tissues are pulped, chopped, or minced coarsely beforehand [Zimmermann and Schäfer (1960) describe a sterile meat grinder], predigested with trypsin to remove toxic substances, then digested further either to a monodisperse suspension (e.g., chick embryo cultures) or to a suspension of clumps containing a dozen or so cells (e.g., kidney cultures). Other proteolytic enzymes have been described such as papain (Hoang et al., 1959), pancreatin (Kolesnikov and Gorev, 1958), and collagenase (Hinz and Syverton, 1959); Ledinko (1955) dispersed chick embryo lung by simple pipetting. Collagenase is useful for organs rich in connective tissue, and it is likely that different dispersion methods will yield cell types of differing viral sensitivity. The cells can be used forthwith for the agar cell-suspension method, or allowed to form a primary monolayer, which can be used as such for assays, or allowed to grow, resuspended with trypsin, Versene, or trypsin plus Versene (preferably in absence of Ca\(^{++}\) and Mg\(^{++}\)), reimplanted as secondary cultures, and used as such. Secondary cultures are often preferable to primary ones for their higher yield of cells per gram of original tissue, and for their more uniform nature, but they may be less vigorous. Winocour and Sachs (1960) found that polyoma virus plaques appeared sooner on primary than on secondary mouse embryo monolayers, a factor of importance in such a slow-growing virus; mouse species was not important. The age of the source animal may have an influence, cells from young or embryonic individuals being generally more sensitive than adults. Osterhout and Tamm (1959) reported that the sensitivity of individual human amnions to herpes virus vary widely. Monkey kidney cells could be stored at \(-75^\circ\) and re-used without change in sensitivity (Stulberg et al., 1959).

The intact animal as source has the advantages of easy access to large cell numbers, fair reproducibility of cultures and freedom from the complete loss of cell source by microbial contamination. It has the disadvantages of giving cultures frequently contaminated with wild viruses (except for human kidneys; Hsiung, 1959a), requiring animal house facilities and preliminary dissection, occasionally being very expensive, and usually giving cultures containing several obviously different cell types, although this can mostly be shown not to affect their performance. However Sellers (1957) found that calf kidney epithelial cells sensitive to FMD virus were overgrown by resistant fibroblasts in ox but not pig serum; Khera and Maurin (1958) eliminated these fibroblasts by storing the cell suspension for 3-4 days at \(0^\circ\). Plaque assay of
a fowl orphan virus (Stoker, 1959) was limited to 3 days by overgrowth of a resistant cell type.

2. Cell Lines Continuously Cultured in Vitro

These cell lines have arisen from occasionally successful attempts to extend cultivation of primary cultures, and may or may not be associated with apparent malignant change. The most frequently used is the HeLa strain, of human malignant origin; its sensitivity to poliovirus is approximately equal to primary monkey kidney, but varies with other viruses. Continuous cell lines of many origins are now widespread, and it appears probable that almost any epithelial culture may yield a line of cells which can be continuously grown and with virus sensitivities similar to the original culture; fibroblastic cultures seem more difficult to cultivate. Continuous lines have the advantages of being generally more reproducible than primary cultures and of being susceptible to cloning so that one can purify the cell type (although the clone so derived is frequently not very stable genetically), of freedom from commensal viral contaminants, and of relative economy in some cases. They have the disadvantages of being frequently difficult to obtain in really large bulk, although this can be achieved (Cooper et al., 1959), of being subject to loss by microbial contamination and other hazards, and of being easily contaminated by other cell lines which are usually difficult to distinguish. Attempts are now being made to put cultivation of these cells on a better footing microbiologically, by storing master cultures at low temperature and devising criteria for distinguishing them. Continuously cultured cells also frequently possess an abnormal metabolism similar to that of cancer cells. They are suitable for both monolayer and agar cell-suspension methods.

3. Cell Lines Continuously Cultured in Vivo

Cells grown in ascitic form in mice comprise such a cheap and bountiful source of cells that it is surprising that more use has not been made of them. The only reports available so far are on their use for growth of EMC virus (Sanders, 1957; Sanders et al., 1958). Their advantages are their abundance (one mouse will reproducibly yield 1 to \(2 \times 10^9\) cells in 10 days), their production directly in monodisperse suspension in the peritoneal cavity in almost pure form, their existence in a considerable number of different cell strains, and their considerable independence from a rigorous sterile technique. Their disadvantages are their host specificity (e.g., it is difficult to grow cells sensitive to primate viruses in mice, although other host animals may be found), the occasional presence of "wild" mouse viruses, dependence on health of the host mouse, and their inability to grow in vitro, including inability to form
monolayers. Their high yield is to some extent counterbalanced by their being required for use in somewhat higher concentration. They can only be used with the agar cell-suspension method.

The actual choice of cells will depend on their availability and the sensitivity to the virus desired to be studied, and Table II gives a list of the cell types found suitable for those viruses examined; other factors may be considered, such as the resistance to interferon-like substances of certain continuously cultivated cell lines (Ho and Enders, 1959). Much better results (bigger plaques or more of them) have usually been obtained after adapting the virus to the tissue by serial passage, by picking a single plaque, or by recombination; many cultures, primary and continuous, can be shown to contain relatively resistant cell types. For obvious reasons, nutritionally less exacting cell lines are preferable.

B. Culture Containers

The container originally used (Dulbecco, 1952) was the Carrel flask, which combined the advantage of optically and culturally suitable glass with the use of a sealed vessel to maintain pH. This type of flask however is expensive and difficult to clean, and makes it difficult to manipulate individual plaques, and the development of an incubator with a controlled CO₂- and water-containing atmosphere (Dulbecco and Vogt, 1953a) made it possible to use petri dishes.

Hsiung and Melnick (1955) returned to a sealed container with a cheap and disposable flat-bottomed glass prescription bottle which could be capped, and current usage is either the petri dish (50, 60, or 100 mm.) or capped bottle or tube (Sommerville, 1959). Specially treated plastic dishes and bottles are now available which will allow formation of a monolayer of cells; ordinary "toxic" disposable plastic dishes can be used with the agar cell-suspension method, however. Both bottles and petri dishes can be used for either monolayer or cell-suspension methods.

The most important objective is a flat base with moderately good optical properties; a nonplane base to a petri dish severely affects accuracy and reproducibility. Advantages of the petri dish are ease of manipulation and cleaning and small volume for incubation; disadvantages are readier contamination and loss of CO₂ and moisture, but these can be met by including antibiotics, omitting glucose and bicarbonate (Cooper, 1961a), or using Tris buffer (Cooper, 1955), and by using a thoroughly humidified cabinet for long-term cell viability (Vogt et al., 1957). The advantage of the sealed bottle is that it eliminates the need for humidifying even up to several weeks' incubation, thus being useful for very slow-growing viruses, and safely contains virulent material; its disadvantages are a large incubation space requirement, tendency of the
agar to slip, and somewhat less easy manipulation; it is essential to include bicarbonate. When they have been compared, bottles and petri dishes are equivalent in sensitivity. Plates and bottles are frequently incubated inverted; Mandel (1958) recommends cutting a notch in the petri dish if condensation forms a liquid seal.

The size of container related to statistical accuracy is discussed in Section V.D. Use of small foci and microscopic counting (Farnham, 1958; Sommerville, 1959), and consequently small containers, is very economical in materials but does not permit ready cloning of plaque isolates; the time factor in their use will depend on the observer's experience.

C. Conditions of Adsorption

Having chosen the cell source, container, and plating method, the next step is to introduce virus to the cells in such a way as to achieve as many successful infections as possible; this stage is one of the most important in obtaining a high efficiency of plating. The agar cell-suspension method does not require a period of adsorption (Cooper, 1955, 1961a; Waterson, 1959a; Macpherson, 1960), and virus attachment appears to occur quite well in the agar-cell layer; this section will therefore be concerned either with dilution media and methods, or with adsorption as applied to cells in monolayers.

1. Dilution Methods and Media

As with bacteriophage, the populations of animal viruses handled tend to be large (up to 10^10/ml. or more), and the only practical way to reduce these to a countable number seems to be to dilute serially, either by 10- or 100-fold steps to near the final level, and then by closer dilutions, perhaps 3-fold. As with bacteriophage (Luria, 1953) it is essential to use a fresh pipet for each dilution. The main source of difficulty at this stage lies in possible losses of virus by adsorption onto glassware or by inactivation. The former is usually negligible if the process is rapid and microscopic volumes are not used, but can be prevented by including small amounts of noninhibitory protein or by silicone - treating the glass (Allison and Valentine, 1960b). Inactivation however can be quite serious, but may be overcome by a variety of devices. Incorporation of purified protein or serum often decreases heat lability and inactivation on freeze-thawing, and "frothing" should be avoided. Skinner and Bradish (1954) warn against inactivation of viruses in strong light, particularly the labile ether-sensitive viruses, and show that serum will protect against this; for detergent-sensitive viruses
(generally also sensitive to ether) detergent-cleaned glassware should clearly be avoided. On the other hand, Cooper (1961a) found that higher titers were often obtained if the ether-resistant poliovirus was diluted through dilute detergent. Lockart and Groman (1958) found a very short half-life of WEE virus at 37°, and performed all dilutions at 0°; Pledger (1960) found that PBS (phosphate-buffered saline, Dulbecco and Vogt 1954a) as diluent gave a low plating efficiency of FMD virus, which was remedied if it was mixed in equal volume with medium containing peptides. Kaplan (1957) diluted herpes virus in 10% heated horse serum in PBS. PBS alone, however, is very widely used as diluent and adsorbing medium, and is generally satisfactory; distilled water is quite satisfactory for dilution of some viruses (e.g., poliovirus) but is lethal for others. Possibly because it is an amine, tris(hydroxymethyl)aminomethane buffer increased markedly the efficiency of adsorption of yellow fever and poxviruses (Porterfield, 1959c; Porterfield and Allison, 1960).

It is usually convenient to adsorb from the same medium as is used as diluent, and during adsorption virus may remain in diluent longer than during dilution. Thus it is advisable to find a diluent allowing both good adsorptive properties and good virus stability.

2. Adsorption Kinetics and Timing

Adsorption rates have been determined for a number of viruses adsorbing onto cells both in suspension and in monolayers, and the process was found to be approximately exponential (e.g., NDV, Rubin et al., 1957; Levine and Sägik, 1956; herpes virus, Kaplan, 1957; poliovirus, Youngner, 1956, Bachtold et al., 1957). However, Valentine and Allison (1959; Allison and Valentine, 1960a,b) pointed out that, since animal cells are larger than bacteria, it is not justifiable to apply the function found adequate for bacteriophage (\( f = 1 - e^{-Kt} \)), and that adsorption in suspension is better expressed by a more complex function

\[
(f = 1 - e^{-Kt-K\sqrt{t}}).
\]

They were able to show that virus particles absorbed with very high efficiency (equal to the theoretical maximum calculated from Brownian collisions) onto glass, but with only 30% efficiency onto trypsinized cell suspensions. The efficient adsorption by glass emphasizes the need for complete coverage of glass surfaces by cells in a monolayer. The rate of adsorption onto glass, however, was much reduced by the presence of protein, while the rate onto cells was not affected (also shown for poliovirus, Youngner, 1956). Adsorption by nontrypsinized cells (monolayers) was more efficient (50%) than by trypsinized cells; it has frequently been
found that trypsinized cells adsorb virus less well (Dulbecco and Vogt, 1954b, WEE virus; Cooper, unpublished observations, VSV and poliovirus). However, the trypsin damage must be rapidly repairable, since freshly trypsinized cells give a high efficiency of plating in the agar cell-suspension method (Cooper, 1955, 1961a).

An entirely different function governed adsorption to cells in a monolayer (Allison and Valentine, 1960b), where \( f = k \sqrt{i} \). It was shown both theoretically and by experiment that adsorption to cells in a monolayer is less rapid than in suspension because the diffusion path was longer; this may account for the higher efficiency of plating of the agar cell-suspension method for viruses approaching the size limit of diffusion through agar. It was also shown that the rate of adsorption to a monolayer should be inversely proportional to the depth of fluid, and it has been a general finding that adsorption rate and efficiency of plating increased with decreasing adsorption volumes. Exceptions to this are those small and stable viruses (e.g., poliovirus, Youngner, 1956; FMD virus, Sellers and Stewart, 1959) which are able to diffuse to and adsorb on cells after the agar has been poured and for which plating efficiency is largely independent of adsorption volume; larger viruses, such as herpes B and vaccinia (Youngner, 1956), are unable to do this, and any such virus which has not adsorbed by the time agar is added will not produce a plaque.

For this reason it has become a common practice with large or unstable viruses to employ as small a volume as practicable (without allowing the monolayer to dry) for as long a time as possible, using lower temperatures (20°–25° rather than 37°) to increase virus stability and to prevent virus growth and release of secondary virus before the agar is added (WEE virus, Lockart and Groman, 1958; NDV, Marcus, 1959; herpes virus, Kaplan, 1957; Waterson, 1958b, Farnham, 1958; vaccinia virus, Furness and Youngner, 1959). On the other hand, Bachrach et al. (1957) found a better efficiency of plating of FMD virus if adsorption was carried out at 39°–43° rather than 26°–29° despite apparently complete adsorption at the lower temperature; this may be related to the effect of neutral red (see Section IV, F.I). Lower temperatures only slow virus adsorption a little (Bachtold et al., 1957; Younger, 1956; Levine and Sagik, 1956). Virus adsorption rate should be, and is found experimentally to be, independent of virus concentration in the low virus:cell ratios used, but is proportional to cell concentration. Larger viruses, as may be expected from their diffusion coefficients, adsorb more slowly than smaller ones, but their efficiency of adsorption (ratio of successful to total contacts) is the same (Allison and Valentine, 1960a).
3. Washing of Plates

It is usual to wash monolayers before virus adsorption with a buffered solution (usually PBS) to remove bicarbonate, which may give a high pH during adsorption, and antibody or other inhibitory substances in the medium, although prewashing lowers yields of vesicular stomatitis virus (Cooper, 1957). Sellers and Stewart (1959) found that such washing doubled the efficiency of plating of FMD virus, apparently without there being inhibitors in the medium. A number of authors also report washing the monolayers after adsorption as a routine procedure, although the object of this is not explained, and the result can only be a lower sensitivity.

4. Medium for Adsorption

Adsorption of virus is dependent on electrolyte, little occurring in isotonic sucrose solutions (Bachtold et al., 1957, poliovirus; Allison and Valentine, 1960b, vaccinia and fowl plague viruses). Ca++ and Mg++ ions are most effective, but sodium chloride alone is quite adequate; cation or sucrose concentrations higher than physiological or isotonic inhibit adsorption, in contrast with infective RNA from the same virus (Ellem and Colter, 1960, Mengo virus). The optimal pH is also physiological, i.e., 6–8, and is rarely critical (NDV, Levine and Sagik, 1956; fowl plague and vaccinia viruses, Allison and Valentine, 1960b; poliovirus, Younger, 1956). In some cases at least adsorption rate is at a minimum at the isoelectric point of the virus, and increases again at still lower pH values. These facts and the small temperature coefficient suggest that the initial adsorption is electrostatic. Lockart and Groman (1958) suggest that bicarbonate inhibits either adsorption or an early stage of infection of WEE virus, in a manner not related to a possible high pH value. Dulbecco and Vogt (1954b) found that some WEE virus could elute from chick cells after adsorption, as also did Lockart and Groman (1958), but several other viruses have been examined for this complication without its being found (NDV and chick cells, Levine and Sagik, 1956; fowl plaque and vaccinia viruses and chick cells, Allison and Valentine, 1960a; poliovirus and ERK cells, Cooper, unpublished results). Marcus (1959) adsorbed NDV to chick cell monolayers in a nutrient serum-containing medium to maintain the cell sheet, since very long (4 hours) adsorption periods were required; this increased the efficiency 2-fold.

5. Optimum Conditions

One may therefore summarize optimum conditions for dilutions as being those performed as rapidly as possible in weak light at 0°, employ-
ing fewest steps and in the presence of noninhibitory protein. The medium used must not be virucidal; PBS may be satisfactory, but the best must be determined for each virus individually. Adsorption is also best carried out rapidly, employing smallest volumes containing the highest cell concentration, at physiological salt concentration and pH and at a relatively low temperature (room temperature may be the best compromise for the cells). Shaking does not apparently increase the rate of cell-virus contact (Valentine and Allison, 1959), probably because of a viscous boundary layer on the cells, but it is desirable to respread the inoculum on monolayers frequently in order to prevent drying out and to redistribute virus trapped in the meniscus. It should be noted that, while care has to be taken to obtain these desirables of adsorption with the monolayer method, the cell-suspension method automatically ensures their presence, a factor possibly contributing to its greater efficiency.

D. Localization of Lesion

It is an essential part of the plaque assay that the infections in the cell layer should remain localized. The original way of doing this (Dulbecco, 1952) was by pouring onto the monolayer molten agar medium which set on cooling, and this has remained the method in common use. All reported uses of the cell-suspension method also rely on agar. Certain alternatives are applicable to particular viruses, and the various possibilities are discussed below and noted in Table II.

I. Agar

Agar is cheap, readily available, not very toxic to cells in culture, and forms a strong gel which can be broken on freezing and thawing or centrifuging in order to extract about half the liquid. It has been used for both monolayer and cell-suspension assays. It has the disadvantages that cells do not behave completely normally in its presence, for reasons not well understood, and that it requires temperatures to melt it which are injurious to medium components; hence medium must be prepared in 2-fold strength and mixed with an equal volume of molten agar. More than one autoclaving may make agar solutions toxic (Dulbecco and Vogt, 1954a).

The agar mostly used is Difco-Bacto, and can be used unwashed for certain cells (e.g., chick embryo) or washed with acetone and water by the procedure of Dulbecco and Vogt (1954a). Prewashed agars (Difco Noble or Ionagar) are on the market and have been used (Gifford and Syverton, 1957; Hsiung and Melnick, 1957a), but occasionally
gave a low efficiency of plating or killed the cells (HeLa cells, Darnell, 1958; ERK cells, Cooper, unpublished observations).

Agar is usually used at those minimal concentrations giving a fairly firm gel (0.9–1.2%) but can be used at 0.6% (Holland and McLaren, 1959) or 0.2% (Hotchin et al., 1960), when it is soft enough to decant, provided that cells are firmly attached to the container (e.g., with calf serum). Agar concentration does not appear to affect plating efficiency of the smaller viruses (30 m$_{\mu}$ diameter), but Marcus (1959) reports that the rate of increase in size of NDV plaques is affected by agar concentration above 1%; herpes virus (Waterson, 1958b) formed very few plaques in agar cell-suspensions and it is mentioned above that several of the medium- and larger-sized viruses (e.g., herpes and vaccinia) do not diffuse well in agar to form plaques after the agar layer is added. There seems reason therefore to accept as a useful approximation Poisson's (1956) estimate of the limiting concentrations of agar for diffusion of viruses, in which 1% agar becomes limiting for 65 m$_{\mu}$ particles, and about 0.7% for 100 m$_{\mu}$ particles. The concentration of agar required to maintain a firm cell layer in the agar cell-suspension method (0.6%) can be considerably less than that of the base layer (1–1.5%), and that as total overlay in monolayers (0.9–1.2%).

While agar is not generally inhibitory to virus growth, nevertheless it does often have some effect, and this is particularly interesting in respect of cell-killing power. Viruses killing cells in liquid media may also kill them under agar, but this is by no means generally true, nor is the converse. Some wild viruses present in monkey kidney cultures ("foamy agents") destroy cultures in liquid but not under agar media (Hsiung et al., 1958). Measles virus (Rapp et al., 1959) gave CPE under agar but no plaques in their hands; many ECHO viruses gave CPE in liquid media but not under agar (Hsiung and Melnick, 1957a) and this did not depend on the serotype of virus but on the strain (sub-type). Farnham (1958) found that agar medium inhibited the spread of HeLa cell infection by herpes virus (plaque formation) although it did not prevent CPE; antiserum did not prevent spread. An extract of agar medium specifically inhibited growth of a small-plaque mutant of poliovirus but not the wild-type (Nomura and Takemori, 1960). Mandel (1958) suggested that the agar overlay partly inhibits the ability of partially neutralized poliovirus to cause infection. Dulbecco and Vogt (1958) found that a large proportion of "d mutant" plaques growing in low bicarbonate contain reverse (d$^+$) mutants which are not selected from the original population, suggesting that the localization under agar enhances the rate of back mutation.

On the other hand, many arborviruses can grow in chick embryo,
monkey kidney, or duck kidney cells without gross CPE, and agar enhances the cell-killing effect (Henderson and Taylor, 1959; Porterfield, 1959a); the same enhancement of CPE was found with an ECHO 11 and a myxovirus growing on monkey and human kidney cells (Hsiung, 1959b). Karzon and Bussell (1959) found that agar increased the sensitivity of canine distemper virus plaque assays two to six times over that of the focal lesion assay.

It is an interesting question as to the cause of the effect of agar. The difference may be purely a mechanical one in that convection is eliminated and the monolayer may develop different physiological conditions from those in liquid media (e.g., become more anaerobic or acid). Cooper (unpublished observations) found that medium extracted from nutrient agar gels did not support growth of ERK cells well, and this effect was eliminated by mixing with complete medium; liquid from frozen-and-thawed agar in distilled water was not toxic, and extra magnesium plus a little calcium almost removed the inhibition in liquid medium, but did not cell growth under agar. Extra calcium in the overlay increased poliovirus plaque growth rate while extra magnesium had no effect (Cooper, 1961a). It is therefore conceivable that agar may be removing some substances, toxigenic or otherwise, from the medium, perhaps by ion exchange; it is probable that the causes are multiple.

There does not appear to have been much effort to justify the assumption that agar does in fact localize the lesion (i.e., completely prevent the formation of secondary plaques), although it probably does since plaque count (but not plaque size) ceases to increase after a certain time. In one case (poliovirus, Cooper, unpublished data) loopfuls of high-titer virus when placed on uninfected plates gave 2-3 mm. artificial plaques in 24 hours and no satellites after 5 days, when the “plaques” were 30 mm. in diameter.

2. Methylcellulose

Methylated cellulose forms a viscous solution in the cold which gels at 37°. Hotchin (1955) used this gel to localize plaques of fowl plague virus on chick embryo cells, and found it had several useful properties: it reliquified on cooling, thus being easily removable; it did not fluoresce under ultraviolet light, thus being useful for fluorescent staining of plaques; it was simpler to handle than agar once made up; it was not apparently toxic. It had some disadvantages, however: plaques developed more slowly than in agar, solutions of methylcellulose media were more difficult to prepare than agar, its gel was weaker, and it inhibited growth of explants more than did plasma clots. Cooper (unpub-
lished data) also found it to reduce markedly the efficiency of plating of poliovirus, but apart from these difficulties it remains a potentially valuable method for certain applications. Rapp et al. (1959) used methylcellulose to obtain foci of measles virus, which were stained by immunofluorescence (see Section IV, F,2).

3. Plasma Clot

The use of gelled plasma is traditional in tissue culture, and Gey and Bang (1951) and Noyes (1953) used it to obtain microfoci or plaques of EEE and vaccinia viruses, respectively. Ledinko (1955) also used plasma to obtain small plaques with influenza viruses, as did Takemori et al. (1955) with Rift Valley fever virus. However, there does not seem much advantage in the use of plasma compared with agar, as the former is expensive and does not give a very firm gel, and this may explain its recent neglect. Mandel (1958) reported that plasma clots gave the same plating efficiency as agar for poliovirus in HeLa cell monolayers. However Underwood (1959) maintained HeLa cells for more than 8 days in plasma clots to get measles plaques; plasma does have the advantage of providing a more "physiological" substrate than agar, and it may be a worthwhile expedient for cells which are difficult to culture, or for long-term assays where the cells are difficult to keep in good shape.

4. No Deliberate Localization

As mentioned above, several of the larger viruses are self-limiting in spread and produce discrete foci without addition of any gelled substrate (e.g. vaccinia and ectromelia viruses, Noyes, 1953; Hanafusa et al., 1959; Postlethwaite, 1960; herpes simplex virus, Farnham, 1958; herpes B virus, Black and Melnick, 1954, 1955; canine distemper virus, Karzon and Bussell, 1959). Some smaller viruses can also be observed to make countable foci which remain local for the first day or so (Sommerville, 1959, ECHO and polioviruses; Parsons and Tyrrell, 1961, common cold virus). The poliovirus foci were more diffuse than those of the ECHO virus, which consisted of round cells that expanded and coalesced. Rather interestingly, the common cold foci (Fig. 1) were produced under roller-tube conditions. The common cold virus would only grow at lower temperatures (33°) and poliovirus is not readily released from cells at these temperatures (Roizman, 1959) although it grows well, and it may be a general rule that certain viruses will produce microfoci better at lower temperatures. It would be interesting to know whether antiserum inhibited the spread of these foci. Hotchin et al. (1960) found a certain amount of localization without agar of influenza
virus "plaques" (detected by hemadsorption) which however were rather spread, presumably by convection, into comet-shaped lesions.

A difficulty of plaque assays without agar is the production of secondary plaques caused by release of some newly formed virus. Secondaries are smaller and thus readily distinguished from primaries, and may be largely or entirely prevented by antiserum, but usually the count must be taken before secondaries appear (e.g., Postlethwaite, 1960). Omission of agar makes the use of a nonspecific cell stain easier as the necrotic cells often leave the glass and the plaque appears as a hole in the cell sheet; it also simplifies the technique somewhat and is useful for viruses whose CPE is inhibited by agar, but it does not have general application. Although cell-to-cell contact is implicated for some viruses, the mechanism for others may be by releasing a very small proportion of their yield.

There are a number of other possible ways of restricting spread which have not been reported but may have limited application, for example, the use of rat tail collagen, or gelatin at lower temperatures.

**E. Conditions of Incubation**

After the virus has been introduced to the cells and the cell layer immobilized if need be, the culture is then incubated to allow the initial particles to invade and grow within their cells, and progeny to be released or spread to adjacent cells. At least two nearly complete cycles are always required, and for macroscopic plaques three to four. The
time of incubation is usually determined by the time required for the plaque count to reach a maximum, and should be constant for a given virus-cell system.

1. pH Control

The optimum pH for plaque development depends upon the characteristics of individual viruses, but in general is not critical, frequently being little affected over the range 6.8-7.8. [Unusually sensitive viruses are the $d$ mutants of poliovirus (Vogt et al., 1957), which are very markedly inhibited at pH values less than 6.8 in presence of bicarbonate.] Usually a pH of 7.2-7.4 is aimed at, although it should be pointed out that the true pH of incubation is rarely known. This is because cells in a monolayer under agar become anaerobic and may produce a locally low pH, and also acidic and basic components in the medium will affect the true bicarbonate ion content; the true CO$_2$ concentration in the usual type of gassed incubator can rarely be estimated from the concentration in the gas mixture being passed. The pH value of solutions buffered with bicarbonate is not easy to measure directly because of the volatile nature of the buffer and its high temperature coefficient. Bicarbonate buffer is also inconvenient to work with, as it readily loses CO$_2$ and becomes alkaline, and may cause a low efficiency of plating (Porterfield, 1959c; Cooper, 1961a).

However, bicarbonate-CO$_2$ systems have been most generally used to control pH, and are essential if sealed vessels (e.g., bottles or desiccators) are used or when measuring the efficiency of plating of poliovirus $d$ mutants. Carbon dioxide concentrations of 3-8% are reported; Waterson (1958a) mentioned that traces of H$_2$S or SO$_2$ in the CO$_2$ may cause trouble. Umbreit et al. (1957) have published a useful graph showing the relationship between pH, temperature, and CO$_2$ and bicarbonate concentrations. Sealed culture containers are not generally deliberately gassed before incubation, but open containers (petri dishes) require to be incubated in a CO$_2$-containing atmosphere. Individual dishes may be sealed with tape, but this is more usually achieved by passing a continuous stream of humidified CO$_2$ and air through an ordinary small laboratory incubator (Dulbecco and Vogt, 1954a), perhaps photoelectrically controlled to avoid undue fluctuations on opening the door (Perkins and Hotchin, 1955), or by flushing a large sealed container with a gas mixture (perhaps CO$_2$ and oxygen only, Bubel et al., 1956). This last is inconvenient, however, as lengthy refilling is necessary each time the container is opened; perhaps the most convenient way of operating a sealed container is to use Pardee's buffer (Bellett, 1960), which is an ethanolamine solution capable of main-
taining a fixed CO₂ concentration in the atmosphere above it. One hundred milliliters can be used twice for one 10-liter container. Small sealed containers are useful if several temperatures or CO₂ concentrations are required.

A still simpler procedure, however, it to omit bicarbonate altogether where possible. Tris(hydroxymethyl)aminomethane (Cooper, 1955; Porterfield, 1959a,b,c; Porterfield and Allison, 1960) can be used satisfactorily with some cells (e.g., chick embryo) and may indeed increase plating efficiency, but is frequently toxic or inadequate for others (e.g., ERK, Krebs ascites cells). The main reason for needing a buffer is the accumulation of CO₂ and nonvolatile acid (mostly lactic); if this is avoided by using an open vessel (petri dish) and a substrate which does not permit much nonvolatile acid production (galactose, Eagle et al., 1958) plaque assays can be made without additional buffer (Cooper, 1961a); the pH value remains within 0.1 unit for 3-4 days, and can be measured directly. Moreover, when the agar cell-suspension method is used, cells will be less anaerobic and pH electrodes can be immersed in the cell layer, so that the true pH can be quite accurately known.

Cells in monolayers are rather anaerobic, and the oxygenation of cultures has received little attention. Oxygen concentrations from 2 to 20% have little effect on poliovirus plaque production (Cooper, unpublished observations) but oxygen tension may be a relevant variable for viruses which do not form plaques easily, particularly those viruses growing naturally in epithelial or surface tissues, or in very anaerobic tissues (e.g., brain).

2. Antibiotics

Antibiotics made modern tissue culture feasible, and although there is an argument for not including them, it will rarely extend to plaque assays provided that antibiotics are shown not to affect plating efficiency; the cover provided by antibiotics greatly extends the scale of working. Penicillin and streptomycin (each about 100 units per milliliter) are almost universally included in plaque assay media, and frequently Mycostatin at 25-75 units per milliliter. In the writer's experience cultures can occasionally be lost when using only penicillin and streptomycin, and he feels that there is every virtue in trying not to lose cultures; for this reason a triple antibacterial cover is preferable, and he finds that neomycin (100 μg. per milliliter), in addition to penicillin and streptomycin, has completely eliminated bacterial contamination over a 3-year period. Sellers and Stewart (1959) use additional polymyxin at 100 units per milliliter.
Mycotic contamination is more difficult to counter, and Mycostatin is not very useful in agar medium since it is so insoluble; it is also unstable and cannot be sterilized by filtration. Fungizone (25 µg. per milliliter) is soluble and has been used with success (Cooper, unpublished data) but is unstable; a more useful substance appears to be 4,4'-diamidinodiphenylamine used at 25 µg. per milliliter (Cooper, 1961a), which is soluble, very stable, and a strong antimycotic; it has greatly decreased the incidence of fungal contamination and has no effect on poliovirus plaque formation, although it is toxic to cells in routine culture.

3. Other Medium Components

It is essential to maintain the cells in a viable state during incubation, and this is achieved by incorporating the usual components of tissue culture media, usually with agar. Cells vary in their requirements and it is sometimes possible to employ quite simple media such as salts plus glucose, but usually a nonvirus-inhibitory serum is included, together with amino acids and vitamins (e.g., lactalbumin hydrolyzate and Difco yeast extract) and glucose or galactose.

Chicken embryo extract (Dulbecco and Vogt, 1954b) was originally used, but is generally now omitted as batches are not reproducible and are occasionally toxic. Waterson (1958b, herpes), and Porterfield (1960, loping ill), however, found it helpful for certain viruses. Fractions of bovine albumin can replace serum, but some protein usually appears necessary. Porterfield et al. (1960), growing arborviruses in chick embryo cells, found that the presence of CPE under agar may depend on a particular batch of serum, most batches being inhibitory. Some sera may be antiprotein and are therefore nonspecifically antiviral (Habel et al., 1958); antiviral inhibitors exist which specifically decrease plaque size without neutralizing the virus (Takemoto and Habel, 1959b). Gey and Bang (1951) considered the question of wild viruses in sera and recommended sterilization by ultraviolet irradiation as least harmful. All batches of sera used for plaque assay should be routinely pre-tested for plaque inhibitory properties.

Although generally agar media are made up to the same composition as virus growth media in absence of agar, the assumption that the actual concentrations of nutrients available to cells is the same may not always be justified. Cooper (1961a) found that the calcium concentration allowing maximum rate of poliovirus plaque development was 3–5 times that in Earle's saline. Type 2 adenovirus plaque formation depends on the relative amounts of arginine and glucose (Bonifas and Mullally, 1960).

With the exception of insoluble antibiotics, media can generally be
made up in 2-fold concentration (in absence of agar) and Selas- or Seitz-filtered as a single solution. They are then warmed to 42°-48°, and mixed with an equal volume of molten agar solution (2-fold in distilled water) at the same temperature to form the final medium, which sets on cooling to a moderately firm gel.

Plaque assays taking a long time (e.g., polyoma virus, 3 weeks, Winocour and Sachs, 1960) or involving cell growth (Rous sarcoma virus, Temin and Rubin, 1958) may require regular supplements in the shape of fresh nutrient agar layers at intervals.

4. Temperature of Incubation

This factor appears to have been rarely considered, occasionally not reported at all, and usually given as 35°-37° without evidence that this is best. Manakar and Groupé (1956) form their initial chick monolayers at 41°; the body temperatures of many animals exceed 37° and it is curious that 37° is routinely used for nonhuman tissue. However temperature is probably not critical; for instance good poliovirus plaques may be obtained in the range of 30°-41°, although certain strains of poliovirus will not grow well at or above 37° ("t" poliovirus mutants, Lwoff and Lwoff, 1960; "cold-adapted" poliovirus variants, Dubes and Wenner, 1957; "common cold" virus, Parsons and Tyrrell, 1961). Unless the incubator is very well humidified, the actual temperature in petri dishes, as recorded by an immersed thermometer, may be a degree below the incubator temperature (Cooper, unpublished observation).

F. Detection of Lesion

In order to be counted, the localized lesion once developed has to be readily distinguishable from the background of uninfected cells. For microscopic lesions (Temin and Rubin, 1958, Rous sarcoma; Farnham, 1958, herpes; Rapp et al., 1959, measles; Sommerville, 1959, ECHO virus) this usually depends on characteristic cytological appearance which in turn depends upon the experience of the observer and the uniformity of control cultures. Individuals vary as to how easy they find microscopic counting to be. Originally (Dulbecco, 1952) the macroscopic WEE virus lesions were recognized in the unstained state, as they appeared different from healthy cells (more "opaque" or "grayish") when observed with oblique light against a dark background. This difference can be temporarily enhanced by making the culture very alkaline; in some systems (vesicular exanthema virus on pig kidney monolayers, McClain, personal communication; NDV on chick embryo monolayers, Franklin et al., 1957) plaques can be counted easily without staining.
However, most systems in use require the development of plaque contrast by staining techniques. The stain may be a "vital" one, i.e., taken up by actively metabolizing cells but not by cells inactivated by virus (e.g., neutral red or tetrazolium salts), or taken up by killed cells and excluded by living ones (trypan blue), or a nonspecific stain (crystal violet, carbol fuchsin) for which, however, the virus-killed cells have to be removed by some device; or an immunological stain (fluorescent antibody), or, not really a stain, detected by hemadsorption.

1. Neutral Red

Neutral red, the stain first used (Dulbecco and Vogt, 1953b) has despite its disadvantages remained the most popular, and colors the live cells so that plaques are visible as white or pinkish areas against a red background. It is cheap and in some cases most effective, but is frequently toxic, is affected by light, can reduce efficiency of plating and may fade readily. It is either incorporated in the agar medium which is added before incubation, or is added at the end of incubation, 3–3 hours before counting and to a final concentration of 1/10,000–1/60,000.

Many authors found that incorporation of neutral red gave a lower plating efficiency (20–75%) than did addition after incubation (VSV, McClain and Hackett, 1958; WEE and polioviruses, Darnell et al., 1958; fowl plague virus, Waterson, 1959; FMD virus, Sellers and Stewart, 1959; Pledger, 1960), although the plaque size was not affected. Strain L cells were killed outright (McClain and Hackett, 1958), as were ERK cells (Cooper, 1959). The effect was always on a stage of virus growth later than adsorption, and in the case of FMD virus this stage was passed 70 minutes after adsorption; this explains why overlying FMD virus monolayers had to be delayed for 70 minutes even though adsorption was complete (Bachrach et al., 1957). Neutral red incorporation appears to be the cause of lack of plaque formation by ECHO 2 virus (Sommerville, 1960b); it also inhibited adenovirus type 2 plaque formation (Bonifas and Schlesinger, 1959).

The toxicity of neutral red appears to be due largely to a photodynamic effect, that is the combined effect of light and the dye which individually do not affect poliovirus growth in monkey kidney monolayers (Klein and Goodgal, 1959; Green and Opton, 1959; Gochenour and Baron, 1959); white light filtered through neutral red solutions did not make neutral red toxic. One hundred foot candles for 15–30 minutes with 1/50,000 neutral red has an appreciable effect, conditions such as would be found most of the time in most laboratories; plates should therefore be darkened immediately on pouring and kept away from light.
light for 24 hours. Gochenour and Baron (1959) found that the cells themselves could change and would develop resistance to fresh neutral red after 2 days in the dark; Ginsburg and Kazymov (1959) reported incubation of human amnion with neutral red before adding poliovirus, but did not state their reasons for doing so. Neutral red had a slight photodynamic action on free poliovirus (Opton and Green, 1960), but not enough to account for its effect on plaque formation. Crowther (1960) found that monkey kidney cells exposed to light in presence of very small amounts of neutral red (1/240,000) did not die for 3 days yet produced negligible poliovirus when infected after exposure, and adsorption was not prevented. The susceptible site was therefore presumably present in cells before virus infection. The photodynamic effect can be prevented by exclusion of oxygen or by reducing agents (Heberling and Cheever, 1960).

Virus infection per se is not enough to prevent neutral red uptake, as herpes microfoci (Farnham, 1958) and cells infected with arboviruses in liquid media (Porterfield, 1959a) will absorb the stain. The enhancing effect of agar in encouraging CPE appears to be mainly in preventing infected cells from absorbing neutral red (ECHO 11 and a myxovirus, Hsiung, 1959b; arboviruses, Henderson and Taylor, 1959; Porterfield, 1959a); the cells do not appear to be destroyed, and it is not known whether their damage by virus is purely transitory.

Neutral red staining could be enhanced by adding acid or buffer at pH 4.5 (Cooper, 1959), but the stain soon fades; Gifford and Syverton (1957) and Underwood (1959) placed their plates at 4°-6° for 12-24 hours to enhance staining. Neutral red is not uniformly toxic; some laboratories have no problem, and it is to be wondered as to the precision of the chemical definition always implied by the name “neutral red.” Batches may vary in staining effectiveness, and there is evidence of decrease in effectiveness on storage. The source and purity of the neutral red used is rarely quoted.

2. Other Stains

Presumably because of the unsatisfactory nature of neutral red in many hands, a variety of other staining procedures has been used. If the cells are firmly attached to the glass by use of calf serum, 0.6% agar can be decanted along with poliovirus-killed cells and the remaining cells stained with crystal violet and washed (Holland and McLaren, 1959); similarly, agar can be omitted with vaccinia virus and cells stained with carbol-fuchsin (Postlethwaite, 1960). Plaques show up as holes in the cell sheet. This has the advantages of allowing staining and counting to be done without a wait of several hours, or to be done later at leisure,
but it has the disadvantages of being more tedious for a large number of cultures, of requiring the cells to be efficiently attached, and of having no check for gaps in the cell sheets (areas of dead cells can easily be distinguished macroscopically from gaps by their reflection of oblique light). Trypan blue (0.1%) stains dead but not live cells, and poliovirus plaques in KB cells show up as blue areas on a white background if the concentration and timing are correct (Lwoff and Lwoff, 1960); one removes excess stain after 20–30 minutes, and it is necessary to precalibrate each batch of dye to obtain the right conditions. This gives an excellent control for gaps in the cell sheet, but suffers from the difficulty that one adds a solution which is already colored; the stain is also not permanent, as the surrounding cells die and will absorb stain. These last difficulties do not arise when staining cells with a tetrazolium salt (Cooper, 1959), which is a vital dye and makes plaque counting on ERK cells much easier than does neutral red; the tetrazolium is colorless and is reduced intracellularly to an insoluble colored compound, and so the stain is permanent. This last factor unfortunately means that it cannot be incorporated in the medium; although incorporation of neutral red gives a low plating efficiency it is useful for some purposes.

Dulbecco and Vogt (1953b) suggested the use of fluorescent antibody for viruses which did not form necrotic plaques, and Hotchin (1955) pointed out that Methocel gels did not fluoresce and might be suitable for use with this stain. Rapp et al. (1959) took up these suggestions for measles virus on Hep-2 cells. The methylcellulose was removed, cells were fixed and “stained” with fluorescent antibody, and foci were counted microscopically. They concluded that the method could be used for assay purposes, but was very inconvenient and tedious; in view of the difficulty of completely removing the Methocel, it is to be wondered whether an overlay could not be omitted for this virus.

Another type of virus-specific stain is the use of hemadsorption (Marston and Vaughan, 1960, parainfluenza 3 virus; Hotchin et al., 1960, influenza, mumps, Sendai viruses), in which red blood cells are allowed to attach to the infected cells which presumably develop a coating of virus or virus-specific material after infection. Plaques are localized by means of soft agar, which is decanted in order to allow direct contact between red and tissue cells. This method can only be used for hemadsorbing or hemagglutinating viruses, but does not require visible CPE.

It is intriguing to consider other possibilities of developing visualization of non-necrotic virus plaques, or of plaques formed by slowly developing viruses such as polyoma. A complex complement fixation test might be devised, or acriflavin or other fluorescent dyes might be used (Anderson et al., 1959); it is also likely that such viruses might interfere
with a rapidly necrotizing virus, producing "plaques" of neutral red-positive cells on a white background.

G. Aggregation and "Masking" of Particles

The "particle" measured by plaque assay is that unit indivisible on dilution, and may consist of a clump of infective particles. This will clearly affect sensitivities, and has rarely been considered for individual plaque assay systems. Dulbecco and Vogt (1955) make the point that exponential ("single hit") ultraviolet inactivation of poliovirus is evidence of single dispersion, and Schwerdt and Fogh (1957) observed no clumping of poliovirus particles in the electron microscope. Franklin (1958a) found a linear inactivation of VSV by X-rays, and Rubin et al. (1957) the same for NDV. However, Cooper (1961a) found that titers of poliovirus preparations could frequently be increased by deoxycholate treatment, and Kaplan (1957) found a "multi-hit" heat inactivation of herpes virus, with an increase in titer up to 7-fold on sonic vibration and freeze-thawing; Farnham and Newton (1959) found an exponential heat inactivation of herpes virus. Postlethwaite (1960) found that ultrasonic treatment of vaccinia virus gave a 3- to 5-fold increase in titer, although assays before and after treatment gave a linear plaque count-dose relationship. Thus masking or aggregation can occur, and should be checked for each system by the means mentioned. The degree of aggregation may depend on the cell-disruption procedures employed.

H. Cellular Factors Affecting Sensitivity

It is important for many purposes to obtain as high an efficiency of plating as possible, and the preceding sections have discussed various factors at each stage of the plaque assay which are known to affect its sensitivity. These factors can be summarized as (a) the intrinsic sensitivity of the cells themselves, (b) relative efficiency of the plating system chosen, coupled with (c) efficient adsorption of all virus particles present, (d) adequate incubation, leading to maintenance of cells with a high proportion of viability and the ultimate expression of all primarily infected cells as a plaque, and (e) the efficient detection of all plaques. Attention to these details has in several cases improved efficiency 2- to 5-fold (Marcus, 1959; Darnell and Sawyer, 1959; Cooper, 1961a).

Despite considerable work in rendering all these factors optimal, however, it is rare to find that all possible particles have been detected by plaque assays. Some other system (e.g., the embryonated egg) may give a higher titer for a given preparation (Table II), but even when the plaque assay is the most sensitive available, electron microscopic observations of purified preparations have shown many more physical
than infective particles. Realization of this fact led to the use of the term "plaque-forming unit," rather than "virus particle," as used in earlier papers. The plaque assay is therefore (in common, it must be said, with practically all other animal virus assays) inefficient, even though all possible variables appear to have been controlled. It is proposed in this section to give some consideration to possible reasons for this inefficiency.

A major interest of the writer in considering the plaque assay was to see whether the high particle to infective unit ratio could be due to a plaque assay inefficient for purely technical reasons (i.e., for reasons excluding the intrinsic sensitivity of the cells). Many systems are not adequately investigated, but the conclusion can now be reached that at least for some systems this possibility is unlikely.

Essentially what one is considering are the reasons for the apparently high proportion of nonviable particles revealed by the most sensitive cell system. Taylor and Graham (1959) discuss the sensitivity of plaque assays of poliovirus, and conclude that its low efficiency is either due to poor adsorption, or to content of truly inactive particles; the former can be improved 2-fold with better techniques. The presence of nonviable particles might indeed be due to inactivation during growth or purification for electron microscopy; this is, of course, a particular difficulty with unstable viruses, but Schwerdt and Fogh (1957) have taken some care in harvesting poliovirus after 1-cycle growth, in purifying rapidly under conditions which can be shown not to inactivate virus, and in using the most sensitive cells available (human amnion). Under these circumstances the content of "inactive" particles was considerably decreased, but the best ratio obtained was 36 particles/PFU (Mahoney strain); it might have been preferable to harvest earlier than 12–18 hours to minimize inactivation still more, but it is doubtful whether this would have improved the ratio by more than a factor of two. Thus thermal inactivation after maturation as well as an inadequate assay technique is unlikely to account for the bulk of the excess particles.

There may be three reasons for this apparent excess: (a) a high proportion of the total particles may be truly noninfective, perhaps due to some error in manufacture (if so, they are not prevented from adsorbing to cells, from experiments with isotopically labeled virus; Cooper and Fenwick, unpublished data); (b) a high proportion may not be "dead" but only incapable of proliferating in the particular cell system employed, perhaps by insufficient amounts of the right "trigger"; choice of more sensitive cells may enable them to contribute to posterity; (c) all the particles may be intrinsically capable of growing in the cells used, but with a low probability of success. They may be compared with the seeds of a desert shrub, a few of which fall on good earth but most on stony
ground; alternatively there may be some competitive inactivating process, for example sensitivity to ribonuclease at some critical stage. The possibility of collaboration of particles, perhaps to provide enough enzyme to overcome some bottleneck in virus production, would appear to be eliminated by the single-particle concept (see Section V).

Thus the intrinsic sensitivity of the cell seems clearly implicated as a major unknown factor. Cell sensitivity to viruses is interesting from a fundamental viewpoint, and is of considerable importance in concepts of virus virulence. Quite possibly the above reasons (a), (b), and (c) all play a part; reason (a) seems difficult to test experimentally at present. See note added in proof (p. 378) regarding (c).

It has become a habit to think of virus infection as an “all-or-none” phenomenon, in which adsorption of one infective particle invariably leads to infection, perhaps by analogy with the T-coliphages where this appears to be the case. This may not be justified with animal viruses. There is in fact no reason to suppose that the cell normally remains inert to virus infection; it may defend itself and the infection observed may well be the result of a contest between cell and virus, the outcome of which is a matter of probability and cannot be predicted for any given cell.

Most cells have been selected for study as being the most sensitive to virus infection, but relative degrees of sensitivity exist. Vogt and Dulbecco (1958) selected by virus action a strain of HeLa cells which was more resistant to poliovirus than the parental type, and Darnell and Sawyer (1959) isolated homogeneous clones without contact with virus which had up to 10-fold differences in sensitivity. However, no clones were fully resistant and the differences expressed themselves either as requiring a higher multiplicity of infection to achieve virus growth in the resistant clones, or as a lower efficiency of plating (but with clear plaques) in plaque assays. Adsorption rate differences did not account for these effects, but there appeared to be some deficient or resistant stage between adsorption and the liberation of free RNA (if this is a necessary growth stage), as both types of cell were equally sensitive to infective RNA preparations (Darnell and Sawyer, 1960); other cells resistant to poliovirus (Holland et al., 1959) were so because they were from nonprimate sources and failed to adsorb the virus, but were also equally sensitive to infective RNA. Takemoto and Habel (1959a) found that a HeLa culture was partially susceptible to Coxsackie A9 virus, and virus growth appeared to be balanced by cell growth. Interfering components (Isaacs and Lindenmann, 1957; Cooper and Bellett, 1959) may also play a part. Hsiung (1959a) pointed out that adult tissues possess a type of cellular immunity to viruses not possessed by baby or embry-
onic tissues. Winocour and Sachs (1960) observed that very high multiplicities of polyoma virus were required to infect every cell in a mouse embryo culture.

Like the "asynchrony" in influenza virus infections (Cairns, 1957) there also seems to be a finite random delay in growth of poliovirus amounting to about one hour per growth cycle for single multiplicities which can be eliminated by using a higher multiplicity ("shortened latency," Cooper, unpublished data); this may be termed a probability of reversible delay and similar random effects leading to a probability of irreversible delay may determine whether or not the cell becomes infected at all.

Numerous instances of differences in cell sensitivities to a given virus exist (see Table II); for practical purposes it is frequently possible to increase the virulence of a virus for a given cell type by adapting it by successive growth in that cell type, and the efficiency of plating of a virus strain often depends on its passage history rather than its serotype. Tests for the homogeneity of a cell source are (a) that the production of infective centers be proportional to multiplicity of infection, (b) that plaques be clear and round, and (c) that cells in a population some of which are infected should be killed randomly in proportion to the number infected (Dulbecco and Vogt, 1954a); Drake (1958), however, found that the infection of HeLa cells by poliovirus was not strictly Poissonian.

V. STATISTICAL FACTORS AFFECTING ACCURACY

The preceding sections have been concerned largely with technical factors affecting the sensitivity of plaque assays, that is the proportion of the total virus particles present which register as a plaque. Clearly any uncontrolled variable affecting sensitivity will also affect accuracy, that is the reproducibility of individual assays in a large number of duplicate determinations. These variables will be assumed to be controlled for the present purpose, and this section will consider other factors which affect the accuracy of these assays.

A. The Single-Particle Concept

It is the most important principle of any particle-counting method that every observation scored as positive (e.g., a plaque) be the result of a single particle rather than the reaggregation or collaboration of many particles. If this does not apply it is not a particle-counting method, and all animal virus plaque assays and clonal isolations depend upon this concept. By "particle" is meant that unit which is indivisible on further dilution. Such a "particle" may indeed be a clump of infective units, but the evidence brought against this, when it has been considered, generally...
but not always (see Section IV,G) reveals that the preparation is not aggregated, although a more universal application of these tests is to be desired.

B. Relationship of Plaque-Forming Units to End-Point Dilution Units

While the dose infecting 50% of a number of hosts (animals or tissue cultures) has provided a very useful relative measure, it is an arbitrary unit and does not take account of the real unit (the particle) involved, although it approximates to it. There is a strict relationship between these units; providing there are no errors in dilutions, then at the 50% end-point the distribution of particles among hosts will be Poissonian. Half the hosts receive no particles; by chance a calculable proportion of the half infected will have received more than one particle (average = 1.38 particles). Thus the over-all average, including those hosts not infected, will be 0.69 particles per host, which if both plaque and end-point systems have equal sensitivity, = 0.69 PFU = one ID\textsubscript{50}. Being a smaller unit, titers expressed in terms of ID\textsubscript{50} will be 1.44 \times higher than those expressed as PFU/ml. The end-dilution dose equivalent to one PFU is the ID\textsubscript{63}, when 37% of the hosts are uninfected, and they receive an average of one particle each.

Similar reasoning can be used to obtain the relationship between the statistical accuracies of the two systems. The coefficient of variation of a count of \( n \) randomly distributed units = \( \sqrt{n}/n \), that is, the accuracy increases with the number counted. The unit scored for in plaque assays is the single plaque, which is an all-or-none event; in end-dilution assays, the all-or-none event is the single host, infected or non-infected. At the ID\textsubscript{50} one particle will require on the average about 2 hosts to detect it (one infected and one not); more precisely, 69 particles will require 100 hosts inoculated with an average of one ID\textsubscript{50}. Since 70 plaques per plate is usually about the upper limit of accurate counting, it can be claimed that one plaque culture (e.g., one petri dish) is statistically equivalent to 100 end-dilution hosts (e.g., tube cultures); the precise equivalence will depend upon the number of plaques which can be counted per culture (see Section V,D.). Dulbecco and Vogt (1954a) have calculated that one monkey kidney will produce 15–30 petri dish cultures statistically equivalent to 1200–2400 roller tubes (more tubes than can be produced from one monkey kidney); thus the plaque assay is more economical in cells as well as in time, for an equivalent accuracy.

C. Experimental Support for the Single-Particle Concept

It is important to demonstrate experimentally for all assay systems that a single particle is responsible for infection, and the way this is usually done is to show a linear relationship between concentration of virus inoculated
and plaques observed, that is that plaque count be directly proportional to dose (as it is for bacteriophage, Ellis and Delbrück, 1939, and for chick embryo pocks, Beveridge and Burnet, 1946). The reasoning behind this is that, assuming that \( v \) virus particles are spread out among \( N \) cells according to a Poisson distribution at an average therefore of \( \frac{v}{N} \) particles per cell, then if one particle can form a plaque the number of plaques should be \( N(1 - e^{-av/N}) \), where \( a \) is a constant defining the efficiency of the system. When \( av/N \) is very small compared with \( N \) (the ratio \( v/N \) in plaque assay systems is usually less than \( 10^{-4} \)) the number of plaques approximates to \( av \), i.e., there is a direct proportionality (Dubecco and Vogt, 1954a). If two particles are required to initiate one plaque, then the number of plaques = \( \frac{1}{2} \left\{ (av)^2/N \right\} \); if three, = \( \frac{1}{3} \left\{ (av)^3/N^3 \right\} \); if \( k \),

\[
N \left[ 1 - e^{-av/N} \left( \sum_{i=0}^{k-1} \frac{(av/N)^i}{i!} \right) \right]
\]

and as \( k \) tends to infinity, the number of plaques tends to zero (Franklin, 1958a).

The relationship, where 2 particles are required, is sufficiently nonlinear to be distinguished easily by experiment from the single-particle case, and those requiring higher numbers are even more easily eliminated. The linear relationship between dose and plaque count has been shown for many virus-cell systems, which are indicated in Table II; there are some exceptions which will be discussed in Section V.E.

It may be noted in passing that, although it is universally recognized that one must demonstrate some linear relationship to prove the value of the assay, a surprising latitude is sometimes felt to be available in the choice of the precise coordinates which will allow the points to fall on a straight line.

Additional evidence for the single-particle concept, which is not usually taken into account and which does not assume that the distribution of virus among cells is precisely Poissonian, is the enormous difference in sensitivity which would follow from a comparison of the “single-particle” and “double-particle” hypotheses. In the case of bacteriophage, the single-particle concept is proved by the observation of infective: total particle ratios close to unity; if two particles were required, using as few as \( 10^6 \) to \( 10^7 \) cells for an assay, the most infective preparations would contain less than 1 infective to \( 10^3 \) or \( 10^4 \) “non-infective” particles, yet there are often less than 50 noninfective particles for every infective one. Furthermore, the sensitivity of the assay would vary in inverse proportion to the number of cells used, and this is not the case over a 5- to 10-fold cell con-
concentration range for vesicular stomatitis and poliovirus (Cooper, 1955, 1961a), and for probably most other viruses.

Thus all the evidence is in favor of the single-particle concept. It is possible that a small proportion of cells require more than one particle to infect them in some cell systems, but these would register as completely insensitive in a plaque assay, and would decrease the apparent titer of all doses in proportion to their relative number.

D. Plaque Overlapping

If excess virus is added to a culture, plaques will have multiple origins and the count will be low because adjacent plaques are obscured. This might be avoided by counting when the plaques are still very small, but when following plaque development it is usually found that some plaques appear before others. Apart from the desirability of obtaining maximum sensitivity, this random appearance will affect accuracy, and it is usual to delay counting until the maximum of plaques have appeared although the early arrivals usually have also increased in size (certain assays take so long that some accuracy and sensitivity must be sacrificed by counting prematurely). Because the plaques continue to enlarge after reaching a maximum number, one must count before plaques are obscured by overlapping.

These considerations therefore determine the time of counting, but they also determine the average plaque size at which counting is usually done. Plaque sizes have been indicated in Table II, and it can be seen that they are frequently quite large for the smaller viruses. The time of maximum plaque number is found either by daily counting (although this usually requires incorporation of a stain, which lowers the efficiency of plating, Section IV,F,1), or by determining the distribution of plaque sizes in a sample plate (Cooper, 1961a), when the number in the smallest visible size group (e.g., 0–1 mm.) should be a negligible proportion of the whole.

The largest number of plaques which theoretically allow individual counting has been calculated by Dulbecco and Vogt (1954a), and will depend upon the average plaque size in relation to the culture vessel area (A). When in hexagonal close-packing, n small circles of diameter d occupy an area equal to 0.866nd². If d is the root mean square of the plaque diameter, then the maximum number of non-overlapping circles, N, is A/0.866d². Plaques resulting from V virus particles will be spread randomly over the surface A, and may be considered in terms of the average proportion (V/N) of the N theoretically available circles or sites occupied by a plaque; when V is not negligibly small compared with N, the number of plaques observed = N(1 – e⁻⁷/V). This can be con-
firmed as a good approximation by experiment (Fig. 2c, and Overman and Tamm, 1956).

In view of the usual difficulty of cell supply, one might at this point consider the most efficient use of these cells. One needs statistical efficiency with as few plates as possible, and in a series of say 6 dilution steps, each with a probable error of 7%, the over-all dilution error will be

\[(7 \times \sqrt{6})\% = 17.1\%\]

(Luria, 1953). It is therefore pointless to aim for a statistical counting error of less than 5%, and 10% may have to be accepted; this can be achieved with a total count of 400 or 100 plaques, respectively (coefficient of variation = \(\sqrt{\bar{n}/n}\)). Three replicates may be considered to give adequate cover for individual plating errors, in which case 3-fold dilutions will always give at least one “usable” count (between 33 and 100 plaques per plate), with a total count of 100–300 plaques, and a statistical error always less than 10%. Many plaque assays have in fact been claimed to have “statistical” reproducibility, and the coefficients of variation, when given, vary from 8 to 21%, centering around 15% (e.g., French et al., 1959).

If therefore one wishes to aim for 100 plaques per plate, and if a loss of 5% by overlapping can be accepted, then \(V = 100\), \(N(1 - e^{-V/V}) = 95\), and \(N = 540\). Since \(N = \lambda/0.866d^2\), it can be calculated that the diameter of a petri dish allowing not more than 5% loss through plaque overlapping should be at least 25 times the average plaque diameter; thus the usual 60-mm. petri dish is adequate for plaque diameters up to 2.4 mm. and the 100-mm. dish for those up to 4 mm.

In summary, therefore, a plaque assay can be planned the errors of which will be consistently less than 17% (dilutions) + 10% (statistical) + 5% (overlapping), but it will need 3-fold dilutions, triplicate plating and 100-mm. petri dishes or equivalent if the average plaque diameter at time of maximum plaque number is greater than 2.5 mm.

E. Exceptions to the Linear Relationship

As mentioned above, although most virus-cell systems have yielded a linear relationship between dose and plaques resulting, there have been a number of exceptions.

Rapp et al. (1959), with measles and Hep-2 cells, presented data indicating that lesion number increased more rapidly with dose than was to be expected from the single-particle hypothesis. However, the tube distributions of the virus were stated to be Poissonian, and unusually difficult techniques (methylcellulose media, fluorescent antibody staining, ultraviolet-microscopic counting) were employed, possibly accounting for the unusual dose relationship.
All the remaining exceptions showed the reverse effect, namely that plaque number increased less rapidly with dose than was to be expected from the single-particle concept. In all cases these were shown not to be due to plaque overlapping (which would have the same effect) since the effect appeared below the theoretical plaque number allowing over-

Fig. 2. Nonlinearity of poliovirus plaque count with dose on ERK cells with (A) 63-mm. petri dish (average plaque diameter 2.4 mm., maximum number of non-overlapping circles/dish = 627); (B) 88-mm. petri dish (average plaque diameter 2.8 mm., maximum number of non-overlapping circles/dish = 873). The PFU added are calculated from the observed count at the lower end of the curve. In (A) and (B), the uppermost line is proportional to dose, the middle curve allows for theoretical losses by overlapping, and the lowest gives the observed count. (C) Result of an artificial “plaque” count (5-mm. circles on a 10-cm. square, coordinates plotted from a table of random numbers, maximum number of non-overlapping circles = 463). The upper line is proportional to dose, the middle curve shows the theoretical for non-overlapping circles, and the lowest shows the observed non-overlapping circles. In practice all “plaques” in (C) were countable.

lapping, and comparison with another virus giving the same or larger size of plaque did not show the effect. Sellers (1955), using calf kidney monolayers, found that most FMD virus strains gave a linear relationship, but one strain (Ven. 1) did not. Ledinko (1955), found that all influenza strains in primary chick embryo lung cultures uniformly gave a linear relationship except PR8, which gave a linear curve only on two out of four occasions. Cooper (Fig. 2, A and B) found a similar effect, where with poliovirus type 1 and ERK cells the relationship was nonlinear on
three out of seven occasions; he recommends the use of smaller plaque numbers per plate to avoid this source of unreliability. Ledinko's exceptional PR8 strains gave only partial CPE in roller tubes, whereas the other influenza strains gave complete CPE. McClain and Hackett (1958) found that VSV plaques were proportional to dose in chick embryo and strain L cells, but, with the same strains of virus and plaque sizes, plaques were not proportional to dose in monkey kidney cells and only gave $\frac{1}{3}$ of the titer.

Thus, if one can assume that the same effect is operating in all systems, it seems that the nonlinearity only appears in certain cell-virus strain combinations but among a number of different viruses, and is not uniformly reproducible; it may be correlated with a partial resistance of the cell line involved, and dependent upon its metabolic circumstances. In all cases the vital stain was not added until toward the end of incubation, and therefore does not play a part. A fact of possible relevance is the common observation that plaques may be smaller if they are "crowded"; hence their slower development may result in a proportion being below the threshold of visibility. This may be due to metabolic changes caused by a high local concentration of cell debris, or to interference phenomena.

VI. SPECIAL USES OF THE PLAQUE ASSAY METHOD

Although the plaque assay method as described above has been used largely for assay of free infective virus, small modifications enable it to be used for somewhat different purposes. These purposes and the attendant modifications will be discussed in this section.

A. Assay of Infectious Nucleic Acid

Following the phenolic extraction of apparently free infectious RNA from tobacco mosaic virus, a considerable effort has been devoted to obtaining similar preparations from animal viruses with success in many cases (mostly yielding infective RNA but in one case DNA, polyoma virus, DiMajorca et al., 1959). An obvious need was to apply the plaque assay to this material. Unfortunately this proved difficult initially; although nonsedimentable infectivity sensitive to nucleases (the distinctions from intact virus on which the designation "free nucleic acid" rests in most cases) was clearly shown in animals, its infectivity in vitro (as plaques) was sporadic and not proportional to dose (WEE virus, Wecker and Schäfer, 1957; mouse encephalitis virus, Franklin et al., 1959).

Fortunately Alexander et al. (1958a,b), starting from concentrated and partly purified poliovirus preparations which contained a high con-
centration of NaCl, were able to obtain reproducible plaques from preparations of soluble RNA, and several other authors have now been successful.

The plaques produced by the infectious RNA are characteristic of the genotype of the parental virus and identical with those of the parent in properties (Alexander et al., 1958a,b; Boeyé, 1959); they may be used in identical fashion to construct growth curves similar to those of infective virus (Holland et al., 1960), although the material yielding infective RNA may not itself be infectious (Huppert and Sanders, 1958), so that the curves may not be identical. Intact virus may (poliovirus, Alexander et al., 1958a,b) or may not (EMC, Huppert and Sanders, 1958) yield infective nucleic acid with cold phenol.

The main difference from free virus assays lies in the plating properties of infectious nucleic acid and its relative instability. Since infectious RNA is sensitive to ribonuclease, cells must be washed well to free them from serum. Alexander et al. (1958b) adsorbed infectious RNA onto HeLa or continuously cultured human amnion cells for 20 minutes from a small volume of 1 M NaCl to obtain maximum plaque numbers, and then added agar overlay as for ordinary virus assays; the infection was then resistant to normal serum, and the plaque number was proportional to concentration. Divalent cations may inhibit and phosphate may increase the plating efficiency.

Nearly all subsequent authors have stressed the importance of using a high ionic strength in the adsorbing medium. Boeyé (1959) found that a higher efficiency was obtained with one HeLa clone line if the salt concentration was increased by stepwise washings up to 1.2 M, and then decreased in the same way before overlaying. Some clone lines were more sensitive to infectious RNA than others. Holland et al. (1959) added Versene before phenol extraction to minimize inactivation by metal ions. Adsorption is generally carried out at room temperature but can be done at 37°; short times (10–20 minutes) are preferred to minimize damage to the cells by the high ionic strength. For poliovirus infective RNA assays (Koch et al., 1960), the effect of high salt concentration was on the cells, not the RNA; monolayers were prewashed with 0.6 M NaCl buffered at pH 7.6 in absence of Ca++ and Mg++. Hypophosphorous acid should be absent from the phenol, which itself need not be removed if the inoculum is diluted 100-fold or more. Phosphate did not affect the extraction, and 0.2 M phthalate improved it. The plating efficiency increased continually with ionic strength, and the limit appears to be set by the resistance of the cells to such adverse solute concentrations (1–1.5 M): cells in monolayers tended to resuspend from the glass. The plating efficiency of virus itself was not increased by these
conditions; also, unlike its effect on intact virus, high pH greatly increased RNA plating efficiency in NaCl, pH 8.2 being the best. Thus there were three important factors: pretreatment with and adsorption from hypertonic salt, and high pH. Plaques were proportional to RNA concentrations.

Ellem and Colter (1960, Mengo virus and L cells) found similar conditions to be optimal, preferring however to avoid the problem of cell resuspension from monolayers by infecting the cells in suspension at 37°, and then assaying for infected cells on fresh monolayers (see Section VI,B). Optimal sucrose (0.7 M) was more effective than optimal NaCl. They suggest that the effect of hypertonicity was not to "precipitate" the RNA onto the cells, but was more likely to inhibit ribonuclease or nonspecific cellular combinations.

Findings generally similar to those of Koch et al. (1960), but differing considerably in detail, were presented for poliovirus RNA and HeLa cells by Holland et al. (1960). They overcame the difficulty of cells resuspending from monolayers by attaching cells firmly by growth in 20% calf serum. As adsorbent, 2 M Mg++ was considerably more effective than NaCl or sucrose, and the SO₄²⁻ ion was much less toxic than Cl⁻. Small amounts of Versene were included. Adsorption was from 0.1 ml. at room temperature for 12–15 minutes with rocking (the timing being critical) followed by washing and overlaying. The pH had no effect between pH 6 and 8, and adsorption was slowed at 4°. The efficiency was 0.1–0.3% of the original virus preparation, and a linear relation of dose to plaque counts was claimed, although the data as presented actually showed a relation more akin to the hypothetical 2-particle curve presented by Franklin (1958a), indicating that the titers obtained will depend upon dilutions used. The method appeared very reproducible, and the RNA adsorbed very rapidly whatever the salt concentration, which appeared merely to increase the effectiveness of the combination.

Bachrach (1960) obtained a reproducible assay, with Poissonian plaque distribution and linear dose-plaque relationship, by adsorbing FMD virus RNA from Ca- and Mg-free PBS, but the efficiency was approximately 10⁴ that of the poliovirus assays; high salt concentration effects were not reported. Like the other authors quoted he found that adsorption of infective RNA was very rapid, being complete in 1 minute; unlike the assay of intact virus, in which overlaying with neutral red media required a delay of 60–90 minutes (Bachrach et al., 1957), infective RNA assays were independent of neutral red. Thus an interesting implication is that infection with soluble RNA bypassed the blockage induced by neutral red (Pledger, 1960).
B. Assay of Infected Cells

It is frequently necessary to determine the content of cells in a suspension able to liberate virus ("infective centers"), and this can be done by methods similar to those used for free virus. A number of minor differences need to be borne in mind, however; infected cells are more delicate than free virus, and their ability to adsorb and produce virus is easily damaged by trypsin, Versene and strong pipetting through narrow pipets; they should be gently diluted in serum-containing medium using wide-bore pipets with avoidance of frothing. Dilutions should be rapid to avoid sedimentation of the cells, and be kept at ice temperature to minimize firm attachment to glass and development of virus growth; silicone- or paraffin wax-treated glassware is also desirable. The control should be performed of centrifuging for 5 minutes at 1000 g after final plating of the cells and assaying of the supernatant to ensure that free virus is negligible; a most essential point is to add the agar before any cells have been allowed to release new virus (i.e., at least ½ hour before the end of the latent period).

Having obtained the final dilution, infected cells can be plated like free virus onto monolayers (30 minutes in PBS at 37°, Dulbecco and Vogt, 1954b). This is satisfactory for small stable viruses such as poliovirus, but it gives low plating efficiencies (20-30%) with larger unstable viruses, as the cells are resuspended on adding the agar and become fixed at points distant from the monolayer, and their yield of virus may die before reaching the monolayer. The simplest way of overcoming this difficulty is to use the agar cell-suspension method (Cooper, 1955), which improves the efficiency of plating of infected cells 3- to 4-fold by achieving very short diffusion paths. A variety of other devices have been employed to gain the same effect. One can incubate the cells for an extended time (4-16 hours) at 24-37° in nutrient medium to allow the cells to attach more firmly to a monolayer (e.g., vaccinia virus, Furness and Youngner, 1959; Rous sarcoma virus, Temin and Rubin, 1958; polyoma virus, Winocour and Sachs, 1960), but this is only suitable for those viruses with a long latent period. Alternatively one can set the infected cells in a small volume of agar (about 1 ml.) over a monolayer, thus achieving a shorter diffusion path, before adding the larger volume of nutrient agar necessary to keep the cells viable (poliovirus, Drake, 1958; polyoma virus, Winocour and Sachs, 1960; herpes virus, Kaplan, 1957). Cells are not harmed as infective centers by a short sojourn at 43°-44°. Infective centers in monolayers which will not form plaques can be “developed” by overlaying with an agar cell-suspension (Frank-
Infective centers should be proportional to virus dose, but may not always be so (Rous sarcoma virus, Temin and Rubin, 1958); Drake (1958) finds that the number of infective centers does not quite follow a Poisson distribution of poliovirus.

C. Plaque Inhibition Tests

Substances inhibiting virus growth (e.g., antisera or interferon) can be estimated rather accurately by using a method analogous to the cup-plate assay for antibiotics, although its sensitivity is lower than end-point methods. In this method a virus inhibitor is placed in a container on an agar substrate and allowed to diffuse during plaque development; if the agar substrate also contains a cell culture through which virus growth is spreading at the correct rate, then each cup will produce a circular zone of protected cells (Fig. 3), the diameter of which will increase with inhibitor concentration. The plates are stained in a similar fashion to plaque assays after an appropriate incubation.

Several methods of doing this have been published. The simplest (DeSomer and Prinzie, 1957) is probably to soak inhibitor (in this case poliovirus antibody) into filter paper disks which are laid on infected monolayers overlaid with agar, and Farrell and Reid (1959) developed...
this into a method as precise (standard error 14%) as the metabolic inhibition test, yet more simple and sensitive. However, the diffusion time with monolayers is limited because of the need to maintain cell viability and compete with virus growth, and Burt and Cooper (1961) have taken advantage of the fact that cells can be omitted from agar cell-suspension assays until diffusion (in the base layer) has proceeded to an optimal extent. Use of this method was more convenient and increased sensitivity 10-fold over an optimal monolayer method used for comparison. Porterfield (1959b) used a monolayer arborvirus plaque inhibition method to assay interferon preparations contained in small hollow beads; he found (Porterfield, 1960) that this method was also very useful for rapid semi-quantitative serological typing of large numbers of arborvirus preparations.

Besides the need to obtain maximum zone sizes (by use of large volumes of inhibitor, long diffusion times, and shallow agar) the most important factor is the ratio of virus to cells; if too much virus is added, the zones are small and pale, and if too little they are large but with very ragged edges which render accurate measurement difficult. This factor fortunately is not particularly critical. The diameter or the square of the diameter of the inhibition zone are both approximately proportional to the logarithm of the inhibitor concentration. Under its best conditions the method is at least as accurate and reproducible as are other methods for determining virus inhibition, but may be less sensitive; it is however by far the most rapid and convenient, and large numbers of samples can be handled.

Another type of plaque inhibition test has been used by Kaplan et al. (1960) to assay the infectivity of rabies virus. It is essentially an end-point method, determining the dose of rabies virus which will reduce the number of WEE virus plaques by interference, and is not very sensitive but is accurate and useful in view of the absence of CPE of rabies virus.

**D. Genetic Studies**

1. **Clone Picking**

A major aim of the plaque technique was to obtain clones of virus which were genetically pure, i.e., obtained from a single parental particle, and this has been achieved on many occasions. Provided that no variation has occurred during development of a plaque (Dulbecco and Vogt, 1958), this goal is ensured by the single-particle concept, but a few technical points have to be observed. The assay should give a linear dose-response relation, and particles must not be aggregated. If mono-
layers have been used, it is important to wash after adsorption to remove nonadsorbed particles. After overlaying, no liquid must fall on the surface so that condensation must be avoided or plates incubated inverted; stain cannot be added after incubation, and must, therefore, either be incorporated into the medium or preferably omitted altogether. Plaques must have nonmultiple origins (no overlapping), and must therefore be well spaced; poliovirus has been found 3-4 mm. from the rim of a plaque (Dulbecco and Vogt, 1954a). Thus one should use less than 10 (preferably only 1) fully developed plaques per dish, at least 10 mm. from a neighbor, and pick with a Pasteur pipet only a 1-mm. cylinder from the center of the plaque. Plaque purification should preferably be repeated twice. Virus clones cannot satisfactorily be isolated from foci obtained in liquid media, unless it is certain that only one focus is present in the container (i.e., at limiting dilution).

2. Plaque Morphology

Plaque techniques have been used to characterize and separate viruses, either of different species or of mutants and related parental strains. These characterizations may be classed as based upon (a) plaque morphology, (b) rate of plaque development, (c) inclusion of specific antiserum, or (d) choice of different cell types, used in separate containers (use of deliberately mixed host cells, giving “turbid” plaques, does not yet appear to have been reported, although some cell sources happen to give turbid plaques). The last two involve considerations which have been discussed above, but the first two will be examined here.

A number of authors (Sellers, 1957, FMD virus; Hsiung and Melnick, 1957a,b, enteroviruses; McClain et al., 1958, vesicular exanthema virus) have described considerable variations of plaque morphology among different types or subtypes of virus, which are generally genetically stable provided that the growth conditions are kept constant. Thus morphology depends on the particular virus, host cells, and cultural conditions used. An exception appears to be the wide variation in plaque size of certain viruses which is not reduced by isolating clones from large or small plaques (discussed in Section VI.D.3). Another qualified exception is that plaque morphology may depend upon passage history (FMD virus, Sellers, 1957, Sellers and Stewart, 1959; West Nile virus, Bhatt and Work, 1957), early passages giving small irregular plaques and later passages large well-rounded ones, suggesting that plaque morphology may not always be very stable genetically and therefore is to be used with care in genetic or classification studies. This also applies to host cell specificity.
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The plaque characters observed are (a) size ("large," "medium," "small," or "minute"), (b) rate of development (not necessarily correlated with size), (c) boundary type ("sharp" or "diffuse"), (d) shape ("round" or "irregular"), (e) center ("clear," "hazy," "opaque," or "turbid"). These factors can all vary independently, and can be different in different cells or cultural conditions for the one virus.

There seem a priori to be many factors which might affect plaque size, which itself depends on the rate of diffusion and the adsorption efficiency of the virus, the rate of intracellular growth and maturation and the rate of release, and the rate of inactivation before and after release (all in presence of agar rather than in liquid medium). Critical concentrations of neutralizing antibody can reduce plaque size without reducing plaque number, and certain plaque size-reducing sera (Take-moto and Habel, 1959b) do the same over a wider range of concentrations. A large plaque variant of vesicular exanthema virus was released faster and was more stable than a minute plaque variant (McClain and Hackett, 1959), although the former was adsorbed more slowly; pseudorabies virus (Kaplan and Vatter, 1959) adsorbed faster than did herpes virus, was more thermostable, had a shorter latent period, and gave higher yields, and accordingly gave much larger plaques than herpes virus; plaque size was correlated with burst size for the three poliovirus types (Youngner, 1956). On the other hand, VSV gave as large plaques in L cells as in chick embryo and HeLa cells (McClain and Hackett, 1958), yet yields from L cells in liquid media were 1–10% of yields from other cells; thus growth in agar and liquid media here do not correspond. Sommerville et al. (1958) investigated the growth rates on monkey kidney in liquid media of four ECHO viruses giving large circular, medium-sized irregular, very small irregular, and no plaques, respectively, but found no correlation between plaque type and growth rate or yield per cell in liquid media, although adsorption rates may account for most of the differences found (Sommerville, 1960a).

Avirulent strains tend to produce smaller plaques (FMD virus, Sellers et al., 1959; poliovirus d and s mutants, Vogt et al., 1957; Hsiung and Melnick, 1958; NDV, Marcus, 1959; vesicular exanthema virus, McClain et al., 1958). The d mutants of poliovirus are more repressed in acid conditions in the presence of bicarbonate; this effect is enhanced by deeper agar layers, but is not shown in liquid medium, and there are various degrees of d attenuation. It seems that the cells' susceptibility changes after one cycle of virus growth under agar, but otherwise this interesting effect remains unexplained. A small plaque variant (m) of poliovirus is not obviously different from its wild-type parent in gross CPE or growth rate in ordinary liquid medium (Nomura and Take-
mori, 1960), yet is specifically inhibited by an extract of agar medium 
(Takemori and Nomura, 1960). Horse serum inhibits release but not 
growth of polyoma virus (Winocour and Sachs, 1960), thus giving small 
plaques or none at all (Dulbecco and Freeman, 1959). A number of 
plaque type variants of poliovirus (Dubes, 1956; Dubes and Wenner, 
1957) were either “cold adapted” (giving smaller plaques at 36° and 
larger at 30°), “cystine independent” (larger plaques than wild-type in 
absence of cystine), or “cystine inhibited” (excess cystine gave turbid 
plaques).

Rate of development will often be correlated with plaque size, but 
may not in the case, for instance, where release occurs although CPE 
is delayed; an “explosive” development may occur at late times (e.g., 
lymphocytic choriomeningitis virus in chick cells, Benson and Hotchin, 
1960), giving larger plaques than those whose development was more 
steady. Arboviruses from groups A, B, and C gave morphologically 
similar plaques in chick embryo, duck kidney, and monkey kidney cul­
tures, but could be distinguished by their time of appearance in these 
cells (Henderson and Taylor, 1959, 1960). Levine (1958) could count 
WEE virus plaques at 48 hours in presence of NDV, as plaques of the 
latter virus did not appear until later.

The remaining factors (boundary type, plaque shape, clarity of 
center) probably result in part from cellular heterogeneity; “hazy cen­
tered” plaques can be observed to contain apparently unaffected cells. 
Resistance in cells is not absolute (Vogt and Dulbecco, 1958; Darnell 
and Sawyer, 1960), and a proportion of cells requiring a higher multi­
plicity to infect them may give plaques with diffuse or irregular out­
lines. A large random delay in onset of CPE (in contrast, for instance, 
with cells which are killed synchronously), or a gradual onset in con­
trast to a “burst-like” one, or production of interfering components at 
high multiplicity, may also have the same effect.

Foci in absence of agar may also vary in morphology (Sommerville, 
1959), ECHO viruses types 1 and 7 and poliovirus giving larger and 
more diffuse foci than ECHO viruses types 2 or 11 in monkey kidney.

3. Plaque Size Variation

A number of authors (e.g., Dulbecco and Vogt, 1954a, Hsiung and 
Melnick, 1957b, Cooper, 1961a, for polio, ECHO, and Coxsackie viruses; 
Buthala, 1960, and Macpherson, 1960, for a fowl orphan virus) have 
commented on the wide variation of plaque size produced by some 
viruses. Beyond saying that plaque sizes still varied after cloning and 
that variation was not due to differences in time of adsorption, since 
washed monolayers or cold-adsorbed infective centers gave much the
Fig. 4. Comparison of the distribution of 4-day plaque sizes of (a) vesicular stomatitis virus in chick embryo cell cultures, (b) poliovirus in ERK cell cultures. Vesicular stomatitis virus is sensitive to ether and is released continuously, poliovirus is not.
same effect, it was stated that the reason was not known. Comparison of comments on size variation and of published photographs of plaques, however, (Dulbecco and Vogt, 1953b; Levine et al., 1959; Macpherson, 1960; McClain and Hackett, 1958, 1959; Hsiung and Melnick, 1957b; Sanders, 1957; Pereira and Pereira, 1959; Henderson and Taylor, 1960; Waterson, 1958a) suggested to the writer that plaque size variation was greater for those viruses insensitive to ether than for those ether sensitive. This difference is exemplified in the photographs of Fig. 4. There is a correlation between mode of release of these two classes of virus and their reaction to ether (Franklin, 1958b), sensitive ones being released continuously and resistant ones discontinuously in a “burst.” One “bursting” virus at least (poliovirus, Howes, 1959) has a large random delay in time of release. The line of evidence is admittedly rather tenuous and plaque size must depend on many factors, but it is nevertheless suggested on this basis that large nonhereditary plaque size variations may be the expression of a large random delay in release characteristics, and are to be expected for viruses maturing internally or otherwise spending a long time associated with the cell.

E. Diagnostic Uses

It has been mentioned that the plaque technique is valuable for primary isolation in some cases (Porterfield et al., 1960; Hsiung, 1959b) as the agar overlay enables CPE to be developed in cultures which would otherwise not show it. Hsiung and Melnick (1957a,b) have also used the method extensively in place of the more usual tube isolations for cell-killing viruses, and Porterfield (1960) describes a field laboratory application in simple typing of arborvirus isolates. The purpose of this section is to point out that much diagnostic virology at present compares with bacteriology before the advent of solid media, and that more information is to be gained by the development and further application of solid medium techniques in virus isolation, either as “spot tests” for CPE (when a large number of samples can be tested on one plate), or spreading mixed samples to isolate “colonies” (Cooper, 1955), or by plaque inhibition tests for typing or detecting noncytopathogenic viruses by interference.

VII. Methods for Individual Viruses

Table II gives an outline of the methods available for many viruses, representative of all the major groups known. An attempt has been made to select those reports which contribute to technique rather than to list those papers which use existing techniques; while not claiming to be exhaustive on the methodology, it is hoped that there have not been
**TABLE II**

The Methods Available for Plaque Assay of Animal Viruses

In the second column, P or S = primary or secondary cultures, M = monkey, K = kidney, CE = chick embryo, hu = human; in the third column, M = monolayer, S = cell-suspension method. The classification implied by the grouping of these viruses is tentative (Cooper, 1961b), and is intended to assist the making of analogies which sometimes helps to resolve difficulties of technique.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cells</th>
<th>Plating system</th>
<th>Mn. diam.</th>
<th>Days development</th>
<th>Relative sensitivity</th>
<th>Plaque localization</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia</td>
<td>P-CE</td>
<td>M</td>
<td>5</td>
<td>Pocks</td>
<td>Plasma slot or none</td>
<td></td>
<td></td>
<td>Noyes (1953)</td>
</tr>
<tr>
<td>P-MK</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Youngner (1956)</td>
</tr>
<tr>
<td>P-CEK</td>
<td>M 0.5-1</td>
<td>3-4</td>
<td>1.6 × egg ID₅₀</td>
<td>Agar</td>
<td>Linear</td>
<td></td>
<td></td>
<td>Wright and Sagik (1958)</td>
</tr>
<tr>
<td>P-MK</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Furness and Youngner (1959)</td>
</tr>
<tr>
<td>L strain</td>
<td>L</td>
<td>2</td>
<td>2-3</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td>Hanafusa et al. (1959)</td>
</tr>
<tr>
<td>P-CE</td>
<td>M 0.5-1</td>
<td>36 hr.</td>
<td>20-25 % of pocks</td>
<td>Agar</td>
<td>Linear</td>
<td></td>
<td></td>
<td>Postlethwaite (1956)</td>
</tr>
<tr>
<td>P-CE</td>
<td>M 3-4</td>
<td>4-5</td>
<td>Pocks</td>
<td>Agar</td>
<td>Tris buffer better</td>
<td></td>
<td></td>
<td>Porterfield and Allison (1960)</td>
</tr>
<tr>
<td>L strain</td>
<td>L</td>
<td>1</td>
<td>2-3</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td>Hanafusa et al. (1959)</td>
</tr>
<tr>
<td>P-CE</td>
<td>M 1</td>
<td>6-7</td>
<td>12 % of pocks</td>
<td>Agar</td>
<td></td>
<td></td>
<td></td>
<td>Porterfield and Allison (1960)</td>
</tr>
<tr>
<td>Cowpox</td>
<td>P-CE</td>
<td>M</td>
<td>3-4</td>
<td>Pocks</td>
<td>Agar</td>
<td>Depends on serum</td>
<td></td>
<td>Porterfield (1960, personal communication)</td>
</tr>
<tr>
<td>Myxoma</td>
<td>P-CE</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpes simplex</td>
<td>P-rabbit K</td>
<td>1-2</td>
<td>4-6</td>
<td>13-94 × pocks</td>
<td>Agar</td>
<td>Used RK-adapted virus strain.</td>
<td>Kaplan (1957), Kaplan and Vatter (1958)</td>
<td></td>
</tr>
<tr>
<td>P-CE</td>
<td>M 1</td>
<td>4</td>
<td>2-3 × TCD₅₀</td>
<td>pock</td>
<td>Agar</td>
<td>Should replace pock method.</td>
<td></td>
<td>Waterston (1958b)</td>
</tr>
<tr>
<td>P-CEK</td>
<td>M 0.5-1</td>
<td>3-4</td>
<td>&gt;microfci HeLa</td>
<td>Egg ID₅₀</td>
<td>Agar</td>
<td>Approx. linear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>M 0.1-0.2</td>
<td>3</td>
<td>50 % of pocks</td>
<td>None</td>
<td>Agar inhibits plaque.</td>
<td>Approx. linear</td>
<td></td>
<td>Wright and Sagik (1958)</td>
</tr>
<tr>
<td>P-he amnion</td>
<td>M 3-4</td>
<td>4</td>
<td>2 × pockts</td>
<td>Agar</td>
<td>Linear</td>
<td>Antiserum needed to prevent</td>
<td></td>
<td>Faraham (1958)</td>
</tr>
<tr>
<td>P-CE</td>
<td>M</td>
<td></td>
<td></td>
<td>Pocks</td>
<td>Agar</td>
<td>Tris buffer better</td>
<td></td>
<td>Porterfield and Allison (1960)</td>
</tr>
<tr>
<td>Virus</td>
<td>Cells</td>
<td>Flattening system</td>
<td>Days development</td>
<td>Relative sensitivity</td>
<td>Plaque localisation</td>
<td>Remarks</td>
<td>References</td>
<td></td>
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<tr>
<td>--------------------</td>
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<td>----------------------------------------------</td>
<td>---------------------------------</td>
<td></td>
</tr>
<tr>
<td>Herpes B</td>
<td>P-MK</td>
<td>M</td>
<td>1</td>
<td>None</td>
<td>Plaque growth not stopped by antiserum</td>
<td>Black and Melnick (1954, 1955)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-MK</td>
<td>M</td>
<td>—</td>
<td>—</td>
<td>Agar</td>
<td>—</td>
<td>Youngner (1956)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-rabbit K</td>
<td>M</td>
<td>2-4</td>
<td>3-4</td>
<td>Agar</td>
<td>Strongly staining peripheral ring (Cooper, 1954, unpublished)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-pig K</td>
<td>M</td>
<td>5-10</td>
<td>3</td>
<td>Agar</td>
<td>—</td>
<td>Kaplan and Vatter (1959)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meserae</td>
<td>P-MK</td>
<td>M</td>
<td>2-3</td>
<td>1-7</td>
<td>Agar</td>
<td>—</td>
<td>Singh et al. (1959)</td>
<td></td>
</tr>
<tr>
<td>Hep-3 strain</td>
<td>M</td>
<td>Microscopic</td>
<td>3-6</td>
<td>3-4 X TCD&lt;sub&gt;30&lt;/sub&gt;</td>
<td>Methyl-cellulose</td>
<td>Sensitivity depends on monkey species (Ishii et al. 1958)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>M</td>
<td>&lt;1</td>
<td>7-8</td>
<td>3.7 X TCD&lt;sub&gt;30&lt;/sub&gt;</td>
<td>Plasma clot</td>
<td>—</td>
<td>Rapp et al. (1959)</td>
<td></td>
</tr>
<tr>
<td>P-CE</td>
<td>M</td>
<td>1-2</td>
<td>10-12</td>
<td>Agar</td>
<td>Linear</td>
<td>Underwood (1959)</td>
<td></td>
<td></td>
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<tr>
<td>P-hu amnion</td>
<td>M</td>
<td>3-4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>De Mayer (1960)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3 strain</td>
<td>W2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Karzon and Bussell (1959)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Adenovirus**

| Type 2             | M       | 9-10 KB best      | Agar              | Depends on relative concentration of arginine and glucose | Bonifas and Schleisinger (1959) |
| Type 3 and 5       | M       | 8                 | Agar              | —                        | Bonifas and Mullally (1960) |
| HeLa, Hep          | M       | 8                 | Agar              | —                        | Wassermann (1960)          |
| Infectious canine  | M       | 1-3               | 5-10 X TCD<sub>30</sub> | Agar                | Linear             | Levine et al. (1959)                        |
| hepatitis          | M       | 1-7               | Agar              | —                        | Singh et al. (1959)       |
| Polyoma            | P-dog K | 3-4               | 14-15 = 1 mouse infectious unit | Agar                | >2 % Horse serum inhibitory | Dulbecco and Freeman (1959) |
| P-pig K            | M       | 3-4               | 14-15 = 1 mouse infectious unit | Agar                | —                  | —                                            |

**TABLE II (Continued)**
### TABLE II (Continued)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cells</th>
<th>Plating system</th>
<th>Days development</th>
<th>Relative sensitivity</th>
<th>Plaque localization</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyoma (cont.)</td>
<td>P or S mouse embryo</td>
<td>M 2-3</td>
<td>11-21 = 1 mouse infectious unit</td>
<td>Agar</td>
<td>Agar medium supplemented at intervals. Linear</td>
<td>Winocour and Sachs (1959, 1960)</td>
<td></td>
</tr>
<tr>
<td>Newcastle disease</td>
<td>P-CE</td>
<td>M 1-2</td>
<td>3 = Egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Agar</td>
<td>Linear</td>
<td>Levine and Sagik (1956)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-CE</td>
<td>M 3</td>
<td>Egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Agar</td>
<td>Counted without staining</td>
<td>Franklin et al. (1957)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-CEK</td>
<td>M 2</td>
<td>0.3-1 X egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Agar</td>
<td>—</td>
<td>Wright and Sagik (1958)</td>
<td></td>
</tr>
<tr>
<td>Hemadsorption Type I</td>
<td>P or S CE</td>
<td>M 2</td>
<td>3 Cell killing = PFU</td>
<td>Agar</td>
<td>Improved sensitivity</td>
<td>Marcus (1959)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FL strain</td>
<td>M &lt;2</td>
<td>2.5 X TCD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Agar</td>
<td>Linear</td>
<td>Deibel (1959)</td>
<td></td>
</tr>
<tr>
<td>Parainfluenza 3</td>
<td>HeLa and other strains</td>
<td>M 1-2</td>
<td>3</td>
<td>—</td>
<td>Agar</td>
<td>Detected by hemadsorption</td>
<td>Marston and Vaughan (1956)</td>
</tr>
<tr>
<td>Sendai, mumps</td>
<td>P-CE</td>
<td>M 2-3</td>
<td>2</td>
<td>—</td>
<td>Dil. agar</td>
<td>Detected by hemadsorption</td>
<td>“comets”</td>
</tr>
<tr>
<td>Unidentified myxovirus</td>
<td>P-bu K</td>
<td>M 1-6</td>
<td>8</td>
<td>Agar</td>
<td>CPE only under agar</td>
<td>Hsing (1959a)</td>
<td></td>
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<tr>
<td>Influenza, various strains</td>
<td>P-CE lung, liver</td>
<td>M 0.3-1</td>
<td>7</td>
<td>2.5% of egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Plasma clot</td>
<td>Fed at intervals. Usually linear</td>
<td>Ledinko (1955)</td>
</tr>
<tr>
<td></td>
<td>P-CE</td>
<td>M 1-5</td>
<td>4-5</td>
<td>0.12-1% of egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Agar</td>
<td>Plaque development depends on virus strain</td>
<td>Granoff (1955)</td>
</tr>
<tr>
<td></td>
<td>P-CEK</td>
<td>M 2</td>
<td>3-4</td>
<td>0.04-1 X egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Agar</td>
<td>—</td>
<td>Wright and Sagik (1958)</td>
</tr>
<tr>
<td></td>
<td>P-CE</td>
<td>M 2-3</td>
<td>2</td>
<td>0.1% of egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Dil. agar</td>
<td>Detected by hemadsorption</td>
<td>“comets”</td>
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<tr>
<td>Swine influenza</td>
<td>P-calf K</td>
<td>M 3</td>
<td>4</td>
<td>10% of egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Agar</td>
<td>Approx. linear</td>
<td>Zimmermann and Schäfer (1959)</td>
</tr>
<tr>
<td>Fowl plague</td>
<td>P-CE</td>
<td>M 3</td>
<td>1-10% of egg ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Methylcellulose</td>
<td>—</td>
<td>Hotchin (1955)</td>
<td></td>
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<tr>
<td></td>
<td>P-CE</td>
<td>M 2-3</td>
<td>3</td>
<td>—</td>
<td>Agar</td>
<td>—</td>
<td>Breitenfeld and Schäfer (1957)</td>
</tr>
<tr>
<td>Virus Type</td>
<td>Virus</td>
<td>Cells</td>
<td>Plat-</td>
<td>Plaque</td>
<td>Remarks</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-------</td>
<td>-------</td>
<td>------</td>
<td>--------</td>
<td>---------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-MK</td>
<td>M</td>
<td>Varied</td>
<td>Agar</td>
<td>Convenient bottles</td>
<td>Huang and McInnich (1955, 1957a,b)</td>
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<tr>
<td></td>
<td>P-MK</td>
<td>M</td>
<td>8</td>
<td>Agar</td>
<td>Agar helps CPE of some viruses</td>
<td>Huang (1959a,b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-MK</td>
<td>M</td>
<td>1.5</td>
<td>None, agar</td>
<td>Rapid and economical (tubes)</td>
<td>Sommerville (1959, 1960b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-hu amnion</td>
<td>M</td>
<td>--</td>
<td>Agar</td>
<td>--</td>
<td>McLaren et al. (1960)</td>
<td></td>
</tr>
<tr>
<td>HA virus</td>
<td>HeLa</td>
<td>M</td>
<td>5</td>
<td>Agar</td>
<td>--</td>
<td>Pereira and Pereira (1959)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-Pig K</td>
<td>M</td>
<td>1-7</td>
<td>Agar</td>
<td>--</td>
<td>Singh et al. (1959b)</td>
<td></td>
</tr>
<tr>
<td>Taihan and</td>
<td>P-Pig K</td>
<td>M</td>
<td>--</td>
<td>Agar</td>
<td>--</td>
<td>Brown and Stewart (1960)</td>
<td></td>
</tr>
<tr>
<td>Tache duo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse encephalomyelitis</td>
<td>L strain</td>
<td>M</td>
<td>--</td>
<td>30-40% of mouse LD50</td>
<td>Agar</td>
<td>Linear</td>
<td>Franklin et al. (1959)</td>
</tr>
<tr>
<td></td>
<td>P-hu embryo K</td>
<td>M</td>
<td>Micro</td>
<td>None</td>
<td>Linear. Incubated at 33\degree</td>
<td>Parsons and Tyrrell (1961)</td>
<td></td>
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<tr>
<td>EMC</td>
<td>Asaites cell strains</td>
<td>S</td>
<td>3-4</td>
<td>--</td>
<td>Agar</td>
<td>Bountiful cell source</td>
<td>Sanders (1957)</td>
</tr>
<tr>
<td>Menge</td>
<td>L strain</td>
<td>M+S</td>
<td>2-3</td>
<td>--</td>
<td>Agar</td>
<td>Combination of suspension and monolayer methods</td>
<td>Elling and Collett (1959)</td>
</tr>
<tr>
<td>FMD group</td>
<td>P-Pig K</td>
<td>M</td>
<td>1-4</td>
<td>2</td>
<td>Agar</td>
<td>--</td>
<td>Sellers (1955)</td>
</tr>
<tr>
<td></td>
<td>S-Pig K</td>
<td>M, S</td>
<td>1-8</td>
<td>2</td>
<td>1.6-2.8 X M</td>
<td>Agar</td>
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### TABLE II (Continued)

<table>
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<tr>
<th>Virus</th>
<th>Cells</th>
<th>Plating system</th>
<th>Days to development</th>
<th>Relative sensitivity</th>
<th>Plaque localisation</th>
<th>Remarks</th>
<th>References</th>
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<tr>
<td><strong>FMD group</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(cont.)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-calf K</td>
<td>M 3-6</td>
<td>3</td>
<td>—</td>
<td>10 × mouse LD₅₀</td>
<td>Agar</td>
<td>Linear</td>
<td>Bachrach et al. (1957)</td>
</tr>
<tr>
<td>P-Pig K</td>
<td>M —</td>
<td>3-4</td>
<td>—</td>
<td>3 × TCD₅₀</td>
<td>Agar</td>
<td>Linear</td>
<td>Khers and Maunin (1958)</td>
</tr>
<tr>
<td>P-mouse embryo</td>
<td>M —</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Saito, quoted by Sellers et al. (1959)</td>
<td></td>
</tr>
<tr>
<td><strong>Infectious bronchitis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-CEK</td>
<td>M 3-4</td>
<td>3</td>
<td>4 × egg LD₅₀</td>
<td>Agar</td>
<td>—</td>
<td></td>
<td>Wright and Sagik (1958)</td>
</tr>
<tr>
<td>Fowl orphan</td>
<td>P-CE liver</td>
<td>Micro</td>
<td>2-3</td>
<td>—</td>
<td>Agar</td>
<td>—</td>
<td>Stoker (1959)</td>
</tr>
<tr>
<td>(originally described as avian lymphoma)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-CEK</td>
<td>S, M 0.5-2</td>
<td>6</td>
<td>10-50 % of TCD₅₀</td>
<td>Agar</td>
<td>—</td>
<td></td>
<td>Levine and Sharpless (1959)</td>
</tr>
<tr>
<td><strong>Lymphoctic chorio- meningitis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-CE liver</td>
<td>S 0.5-5</td>
<td>7-8</td>
<td>8 = 7 × M</td>
<td>Agar</td>
<td>—</td>
<td></td>
<td>Macpherson (1960)</td>
</tr>
<tr>
<td>P-CEK</td>
<td>M 1-2</td>
<td>2-3</td>
<td>—</td>
<td>Agar</td>
<td>—</td>
<td></td>
<td>Buthala (1960)</td>
</tr>
<tr>
<td>P-CE</td>
<td>M 5</td>
<td>12</td>
<td>PFU:TCD₅₀:LD₅₀ = 1:3:4</td>
<td>Agar</td>
<td>—</td>
<td></td>
<td>Benson and Hotehin (1960)</td>
</tr>
</tbody>
</table>
many omissions. However, no attempt has been made to include data reporting simple growth of viruses in tissue culture, although this is a necessary prerequisite for plaque assays and any virus capable of such growth is in theory also capable of being induced to form localized lesions.

VIII. Conclusion

The many practical factors which influence local lesion assays in tissue culture are often interesting for fundamental as well as technical reasons, and have thus contributed to our basic information. It is also clear that so many factors are at work that any new system must be investigated on its own merits to define its particular optima. Nevertheless, as outlined above, much information now exists on which to base such work, and detailed procedures are available for many viruses, covering all the major groups; general rules have emerged which should simplify new developments provided that certain requirements can be met.

The most difficult requirement seems to be to find suitably sensitive cell systems, and considerable interest, both fundamental and applied, lies in finding and perhaps overcoming the cause of a cell's resistance to a virus, which may be very high even for the most sensitive cell system. A practical corollary to such work might be the means to increase a cell's resistance to a virus.

References


**Note added in proof.** With regard to the effect of cell sensitivity on plating efficiency of virus (p. 349), evidence is now available (Joklik and Darnell, *Virology,* 13, 439–447, 1961; Fenwick and Cooper, to be published) that most of the high ratio of physical particles to PFU in poliovirus is due to a low probability of achieving infection among a population of virus particles, most of which are potentially infective.
An Improved Agar Cell-Suspension Plaque Assay for Poliovirus:
Some Factors Affecting Efficiency of Plating

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Accepted October 14, 1960

A method is given for a convenient agar cell-suspension plaque assay of poliovirus using the ERK transformed cell line, and avoiding the need for a CO₂-bicarbonate or other additional buffer by using galactose in place of glucose. A number of factors increased the sensitivity four- to sixfold and made the assay more uniform.

INTRODUCTION

The convenient plaque assay method described for poliovirus is similar to that given for vesicular stomatitis virus (Cooper, 1955), where cells are suspended in agar over an agar base layer rather than spread as a monolayer on glass. When assaying a virus preparation for infectivity, it is often important to obtain the highest possible sensitivity, and a major object of the work described has been to consider some factors that could affect the efficiency of plating (e.o.p.) of animal virus plaque assays. The incorporated improvements result in a four- to sixfold greater sensitivity; other advantages are that before use the cells are kept in suspension so that plates can be prepared at short notice, slightly fewer cells are needed than for the monolayer method, and the needs for bicarbonate buffers and gassing with CO₂ are avoided by replacing the glucose in the medium by galactose. Thus assays may be made in an ordinary incubator, while retaining the particular advantages of petri dishes.

MATERIALS AND METHODS

Cell strain was the Westwood embryo rabbit kidney (ERK) transformed cell line grown either in monolayers or suspended culture as described by Cooper et al. (1959). Cells from the two sources were generally indistinguishable in reaction to virus infection. It was frequently convenient to store cells for plaque assay in a suspension "conservator"; for this a cell suspension (1 to 2 × 10⁶ per milliliter) was stirred for 1-4 days in galactose conservator medium [GCM, a bicarbonate- and glucose-free version of the galactose-containing medium CSV.6 (Cooper et al., 1959)], gassed with air, in a water bath at 28-32°. Cells were taken from this when required, washed once, and used forthwith for plaque assays.

Virus strains used were: Virulent: type 1, Brunhilde and Brunenders; type 2, MEF1; type 3, Saukett and Leon. Attenuated (courtesy of Dr. A. Sabin): type 1, L.Sc. 2ab, KP3; type 2, P.712, Ch. 2ab, KP3; type 3, Leon, 12 ab, KP4. The three types of virus were adapted to ERK cells by one or two serial passages and purified twice by growing from plaques picked from terminal dilutions; during adaptation the t character of the attenuated strains became intermediate between the original seed and the virulent strains. Adequate stocks were built up from these strains by three diluted passages and then partly purified and concentrated by two cycles of slow and fast centrifugation (Schwerdt and Schaffer, 1956), and stored in ampules at −70°.

Plaque assay. Two assay systems were compared: (1) confluent cell sheets adhering to a glass surface (monolayers); (2) cell suspensions in agar medium poured on top of a pre-set agar base layer (agar cellsuspensions). The quantities of medium...
TABLE 1

MEDIUM FOR PLAQUE ASSAY (TWOFOLD FOR DILUTION IN EQUAL VOLUME OF 2.5% AGAR)*

<table>
<thead>
<tr>
<th>Components</th>
<th>In ES2x (g)</th>
<th>In both (g)</th>
<th>In gal 2x (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>12.0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>KC1</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl2</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl2 6H2O</td>
<td>0.4</td>
<td></td>
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<tr>
<td>NH4Cl</td>
<td>0.1</td>
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<tr>
<td>NaH2PO4 2H2O</td>
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</tr>
<tr>
<td>NaHCO3</td>
<td>5.4</td>
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</tr>
<tr>
<td>Galactoseb</td>
<td>—</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>4.0</td>
<td>—</td>
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<tr>
<td>Inositol</td>
<td>0.2</td>
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<tr>
<td>Biotin</td>
<td>0.002</td>
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</tr>
<tr>
<td>Choline chloride</td>
<td>0.002</td>
<td></td>
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<tr>
<td>Folic acid</td>
<td>0.002</td>
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<tr>
<td>Nicotinamide</td>
<td>0.002</td>
<td></td>
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</tr>
<tr>
<td>Calcium pantothenate</td>
<td>0.002</td>
<td></td>
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</tr>
<tr>
<td>Pyridoxal-5'-phosphate</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.00002</td>
<td></td>
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</tr>
<tr>
<td>Yeast extract (Difco)</td>
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<td></td>
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<tr>
<td>Lactalbumen hydrolyzate</td>
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<tr>
<td>Penicillin</td>
<td>500,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(units)</td>
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</tr>
<tr>
<td>Streptomycin</td>
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<tr>
<td>Neomycin</td>
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<tr>
<td>4,4'-Diaminodiphenylamine</td>
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<tr>
<td>dihydrochloride (M &amp; B 938)</td>
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<td></td>
</tr>
<tr>
<td>(or Fungizone, Squibb)</td>
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</tr>
<tr>
<td>L-Glutamine</td>
<td>0.2</td>
<td></td>
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<tr>
<td>L-Glutamic acid</td>
<td>0.6</td>
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<td></td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.2</td>
<td></td>
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<tr>
<td>L-Arginine hydrochloride</td>
<td>0.5</td>
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</tr>
<tr>
<td>Phenol red</td>
<td>0.02</td>
<td></td>
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</tr>
<tr>
<td>Water to</td>
<td>1000 (ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse serum, *</td>
<td>200 (ml)</td>
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</tr>
<tr>
<td>pH</td>
<td>7.3</td>
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</table>

* ES2x = twofold supplemented Earle's saline; gal2x = twofold galactose medium.

b Glucose-free, i.e., <0.3% w/w; Lights & Co., Colnbrook, England.


Virus was diluted in phosphate-buffered saline (PBS); the e.o.p. of many samples was increased by including 2 mg sodium deoxycholate per milliliter in the first step. Samples of 0.2 ml of threefold virus dilutions (ideally containing 20-60 plaque-forming units) were added to 3 × 3/4-inch tubes at room temperature, each containing 1 ml of cell suspension (freshly trypsinized or from "conservators" or suspension cultures) at 5 to 8 × 10⁶ cells per milliliter, then mixed with 1.2 ml of molten 1.25% acetone-washed Difco agar medium at 48° (equal volumes of 2.5% agar and "gal2x", bicarbonate-free, but containing galactose in place of glucose; cf. Table 1), and poured at once on preset agar base layers. No interval for adsorption was necessary. The base layers comprised 10 ml of the same agar medium in 4-inch (88 mm internal diameter) pressed-flat petri dishes, poured on a level surface. After 0.25-8 hours at room temperature, the plates were incubated at 36° in polystyrene bread storage bins without gassing or humidifying. The pH remained at 7.4-7.5 if glucose was absent. Bicarbonate-glucose media (Table 1), used for comparative purposes, were incubated in a continuous flow of humidified 6% CO₂-in-air. After 3-4 days at 36°, plates were stained by 2-3 hours' incubation with 2 ml of 1.5 mg 2-p-iiodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride in 0.9% NaCl (Cooper, 1959); when using either the tetrazolium or neutral red with glucose-free media, however, it was essential to include glucose in the stain (10-20 mg per milliliter of stain). It was necessary to know that the plaque count had reached its maximum; rather than include neutral red in the medium and count daily, which lowered the e.o.p., the criterion was used that a negligible proportion of plaques (<10%) were in the smallest size group (0-1 mm).

No difference in e.o.p. was observed when the cells used were either freshly trypsinized, or maintained in the conservator for up to 4 days, or grown in suspension, provided that at least 90% of the cells did not stain with 0.1% trypan blue. The cells could be
kept, pending use, at 5 to 8 × 10^6/ml without deterioration for 8-10 hours by rocking gently in ice in GCM; to maintain a high e.o.p. they should not be kept for longer than 20 minutes at room temperature without stirring or shaking. High calcium media became turbid after 1-2 hours at 48°, but this did not affect e.o.p. Petri dishes with bicarbonate-free base layers could be stored at 4° in closed cans for 1-2 weeks before use. Half quantities could be used for the 3-inch (63 mm internal diameter) dishes. The media were made up complete, Seitz-filtered (EKS grade pads) in twofold strength, and stored for 1-2 months at 4°.

Diamidinodiphenylamine dihydrochloride (Table 1) (May and Baker, Dagenham, England) was effective against most yeasts and molds encountered without reducing e.o.p. and was preferable to Nystatin in plaque assays for its solubility and stability. Fungizone (Squibb amphotericin B) was also soluble and was useful at the same concentration and at 5 μg/ml in routine monolayer cultures of cells.

RESULTS

Factors Affecting the Efficiency of Plating

A number of innovations in the assay were tried in order to determine their effect on the e.o.p. of the agar cell-suspension plaque assay, and these can be considered in three general categories.

1. Modifications that always improved the e.o.p., often by a factor of 2:
   a. Omission of neutral red from the agar medium at the time of infection
   b. Increase in the Ca++ concentration in the agar medium from 1.5 mM to 7.5 mM
   c. Use of agar cell-suspensions rather than monolayers

2. Modifications that usually improved the e.o.p. somewhat and reduced the day-to-day variations:
   a. Staining with tetrazolium instead of neutral red
   b. Pretesting sera and agar for antiviral and cytotoxic effects
   c. Use of galactose instead of glucose-bicarbonate, with elimination of gassing

3. Factors that could be varied within a wide range without affecting the e.o.p.:
   a. Cell concentration (4 to 20 × 10^6 cells per plate)
   b. pH of medium (6.8 to 7.8)
   c. Adsorption time before mixing with agar and pouring (5 minutes to 3 hours)

The over-all effect of including the modifications in the first two categories was to increase the e.o.p. four to six times over that found with the “standard” method of plating on cell monolayers.

Additional Calcium

Both the glucose-bicarbonate and galactose plaque-assay media of Table 1 contain five times as much calcium chloride as does Earle’s saline. Plaques were much larger in these calcium concentrations and the e.o.p. at 3 days was also 50-100% higher than that of controls (Figs. 1 and 2). Extra magnesium had no effect. The increase of plaque size and e.o.p. with extra calcium was found with glucose-bicarbonate or galactose media, using monolayer or agar cell-suspension assays of both virulent and attenuated virus of all three types and with monkey kidney and ERK cells.

The higher e.o.p. with extra calcium may simply reflect greater efficiency of adsorption, because poliovirus adsorption on monkey kidney cells depends on calcium ions (Bachtold et al., 1957); much calcium may be bound by the agar, so that little free calcium is left for cell-virus interaction. It is difficult, however, to see why extra magnesium does not do the same, since Bachtold et al. (1957) found Ca and Mg ions to be equivalent.
Fig. 1. Size distribution and number of 3-day poliovirus plaques in (left) normal (0.2 g/l), and (right) high (1.0 g/l) calcium chloride concentration, with ERK cells plated as agar cell-suspensions; identical virus inocula and incubation conditions were used.

Fig. 2. Distribution of poliovirus plaque sizes in FRK agar cell-suspensions in normal and high calcium and magnesium concentrations; stained after 3 days at 36°. The single vertical line represents the mean plaque size. A: 1 mM Mg, 1.5 mM Ca (normal); B: 4 mM Mg, 1.5 mM Ca; C: 1 mM Mg, 6 mM Ca; D: 4 mM Mg, 6 mM Ca.

DISCUSSION

Routine monolayer plaque assay (Dulbecco and Vogt, 1954) adapted to ERK cells presented three main sources of difficulty or inconvenience: (1) the cells grew rapidly and the e.o.p. of monolayers was optimal only for about 24 hours; (2) cell suspensions were difficult to free from clumps, which left gaps in the cell sheets simulating plaques; (3) the cells produced much non-volatile acid, necessitating a high bicarbonate concentration and hindering pH control. The CO₂-bicarbonate buffer was also inconvenient because of occasional variations in the gassing system and the need to avoid high alkalinity during manipulation, which may give low plating efficiency and small plaques. Attempts to use other buffers, e.g., phosphate or tris(hydroxymethyl)aminomethane, gave acid plates or low plating efficiencies. The method described overcomes these difficulties and has been in routine use in our laboratory for 18 months.

Experiments with monolayers are also limited to the numbers of preformed monolayers available at the time. This limitation does not apply to the agar cell-suspension method, since cells can be harvested from routine monolayer or suspension cultures and used forthwith; cells may also be conveniently stored in suspension ("conservator") and used as required for periods up to 4 days. Certain other advantages of the agar suspension method are mentioned by Cooper (1955); the disadvantage of requiring more cells than the monolayer method has been overcome for the ERK system.

These conveniences enable more assays
to be performed with the facilities available and also increase the sensitivity of the virus assay. As outlined above, the e.o.p. depends upon a number of factors; an optimal system such as that described compared with, for example, assay as 3-inch monolayers of ERK cells with 50 plaques, the usual calcium concentrations and incorporated neutral red, was more uniform and was also four to six times as sensitive.

This may be the limit of sensitivity of the cell-virus system employed; as regards other systems, monkey kidney cells, when compared directly, were as sensitive as ERK cells and their e.o.p. was improved in the same way as that of ERK cells. It has been shown (Schwerdt and Fogh, 1957; Hsiung and Melnick, 1958) that different cell systems can give different efficiencies of plat­ting, but this will not indicate differences in intrinsic cell sensitivity unless it can also be shown that both systems are under optimal conditions, which may not be the same for both.

It is interesting that galactose supported virus growth well in the absence of glucose and without time for cell adaptation. This contrasts with the observations of Darnell and Eagle (1958), who, however, main­tained the cells in a smaller number of nutrients. Presumably, therefore, some nutrient essential for virus growth or cell survival is not synthesized in the presence of galactose but is provided by the medium described in Table 1. Good plaques were obtained in the absence of galactose in this medium, but not as reproducibly as in its presence.

ACKNOWLEDGMENT

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Studies on the Structure and Function of the Poliovirion: Effect of Concentrated Urea Solutions

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Concentrated urea solutions inactivated poliovirus rapidly at 37°, giving a very reproducible multistage curve followed by an exponential inactivation, with a variable resistant fraction. The damage in urea was largely reversible up to the end of the end of the curve's shoulder; calcium and magnesium ions, ribonuclease, and RNA had no effect on urea inactivation, and PBS, 7 M glycerol, 3 M NaCl, and 2 M guanidine had no effect by themselves, nor did osmotic shock or the cyanate present in urea solutions play a part in the urea reaction. Lower temperatures and concentrations of urea markedly decreased the inactivation rate; the amount of inactivation was at a minimum between pH 6 and 8. Experiments with labeled virus showed that a progressive damage in urea first slightly impaired adsorption and thermal resistance without affecting the integrity of the particle or its sensitivity to antibody, then abruptly ruptured the particle. This caused loss of infectivity and liberated RNA in a non-sedimentable form susceptible to nucleases, while the bulk of the protein shell was still sedimentable. Some RNA occasionally remained infective in the soluble form, and some protein was solubilized with the RNA. This release of nucleic acid suggests sites on the virus particle which may be involved in penetration mechanisms.

INTRODUCTION

In order to obtain information on the structure and components of the poliovirion and their relation to growth processes, it was desired to apply a mild disruptive procedure to virus preparations which might also resemble the effect of the host cell on the virion. Hydrogen bond disruption seemed promising in this respect; preliminary studies of the rate of inactivation of poliovirus by 7.2 M urea showed a multistage inactivation curve which suggested a step-wise dissociation of the virion; this paper presents an analysis of the various products of this dissociation.

METHODS

Typical urea-inactivation experiment. A tube containing 1.6 or 1.8 ml of 8 M reagent grade urea, in 0.1 M barbiturate buffer adjusted to the indicated pH, was allowed to warm in a 37° bath; at zero time 0.4 or 0.2 ml of a warm purified virus preparation in PBS (phosphate-buffered saline) was added to the tube and well mixed, and at intervals (accurate to 10 seconds) 0.2-ml samples were withdrawn and rapidly mixed with 1.8 ml of PBS in an ice bath to stop the reaction (see below) and kept at 0° to 4° until assay. Samples were assayed within 1 or 2 hours after dilution. Unless otherwise stated, all manipulations of virus after urea treatment took place at 0° to 2°. Urea solutions were autoclaved (10 pounds for 10 minutes), stored at 4°, and discarded after a few months as the pH rose on storage. This rise may have been due to production of cyanate (see below), and some experiments were done with freshly made unsterilized urea solutions.

Assay of infectivity of intact virus was by agar cell-suspension plaque assays with ERK cells (Cooper, 1961) and is expressed as plaque-forming units (PFU) per milliliter.
Assay of infective RNA was by a plaque method similar to those of Alexander et al. (1958) and Holland et al. (1960a). Monolayers of ERK cells formed overnight in CSV-6 medium (Cooper et al., 1959) containing 20% calf serum were washed once with each of 0.2, 0.4, and 0.8 M Na₂SO₄ at pH 8. RNA dilutions in 0.1 ml 0.8 M Na₂SO₄ were adsorbed for 15 minutes onto the washed monolayers, which were then washed once in PBS, overlaid with agar, and incubated as usual for plaque assay.

Preparation of purified virus. All virus used was highly purified poliovirus type 1, unlabeled or labeled with phosphate P³² or cystine and methionine S³⁵, and was prepared by a bulk virus growth procedure to be described by Fenwick and Cooper (in preparation). This consisted of growth in ERK cell suspensions containing 10⁷ cells per milliliter and gassed with 95% O₂ and 5% CO₂. The subsequent purification procedure was similar to that described by Hoyer et al. (1959). The purity of the labeled virus was checked in two ways: (1) by comparison of infectivity and radioactivity after centrifugation through CsCl density gradients, and (2) by comparison of adsorption of infectivity and radioactivity by sensitive (ERK) cells which adsorbed virus well at 0°, and by resistant (Ehrlich ascites tumor) cells which did not adsorb poliovirus. In each case >98% of the radioactivity remained associated with the infectivity. Adsorption of radioactivity by ERK cells was almost completely prevented by mild heat inactivation of virus and by low doses of specific antiserum.

Gel filtration. A gel filtration column was formed with 0.4 g dry weight of Sephadex G-75 ("water-regain" = 7.9 ml/gm: Pharmacia, Uppsala, Sweden) slurried in PBS. After settling overnight in a 1-cm diameter column made from a graduated 10-ml serological pipette, the column was washed with PBS under gravity and drained until the solute meniscus met the top of the Sephadex. This was regarded as the start of the experiment for the purposes of measuring effluent volume. The total column volume was noted (V₀ = 4.8 to 5.0 ml); since the volume of solute inside the granules = V₁ = 0.4 x water regain = 3.5 ml, the volume of solute external to the granules can be calculated as V₁ = 3.5 = 1.3 to 1.5 ml = V₀. Virus labeled with P³² was incubated for 6 minutes at 37° in urea (6.4 M, pH 7) and diluted fivefold into PBS at 0°; 1 ml of this dilution was added to the column, and the meniscus was again drained down to the top of the Sephadex. The column was kept at 0°, and the flow rate was about 8 drops per minute. The column was then washed with PBS until the effluent totaled V₀ ml; at this time large molecular weight material was about to leave the column. Equal fractions of about 0.6 ml (9 drops) were subsequently collected and assayed for P³² during the passing of a further (V₀ + V₁) ml of PBS; small molecular weight material began to appear after (V₀ + V₁ − 1) ml of this had passed. In other experiments labeled virus either intact or after extraction with cold phenol was used.

RESULTS

Kinetics of Urea Inactivation

The characteristic inactivation curve. Concentrated urea solutions inactivated poliovirus rapidly at 37° following a characteristic multistage pattern (Fig. 1). Each curve at pH 7 and 8 records results of 3 or 4 experiments. The "shoulders" and slopes of these inactivation curves were reproducible with all batches of virus tested. The upward extrapolation of the curve intercepts the ordinate at a level 10⁷ times the original infectivity, and inactivation after 4-5 minutes was usually exponential to 10⁻⁷ survivors, but in some experiments there was evidence of relatively resistant fractions. These resistant fractions were not reproducibly obtained and will be discussed below in a separate section.

It is concluded that there are at least three products of urea treatment: (1) fully infective material exemplified by the 4-minute product; (2) noninfective product(s) formed largely after 4 or 5 minutes; (3) nonreproducible resistant infective fractions, differing in amount between experiments and noticeable after 6 or 7 minutes in urea. Each of these will be described in turn below.
shows that 7.2 \( M \) urea at pH 8 gave a slightly shorter shoulder than 7.2 \( M \) urea at pH 7, but a similar exponential inactivation rate. A curve at pH 6 (not shown) was substantially identical with those at pH 8, so that infectivity was least inactivated at some pH value between 6 and 8. Reduced glutathione (GSH, \( 10^{-2} M \)) had an effect on the urea inactivation (Fig. 1), and this is discussed further below. Calcium and magnesium chlorides (2 and 1 mM, respectively), 1 mg thymus RNA per milliliter and pancreatic ribonuclease (10 \( \mu g/ml \)), had no effect when incorporated with urea. The effect of urea was not due purely to thermal changes, or to osmotic shock alone, or to a combination of these factors, since 1.1 \( M \) sucrose (pH 4 or 7), PBS (pH 4 or 7), 7 \( M \) glycerol (pH 7), and 3 \( M \) NaCl (pH 7.6) had no effect on infectivity for up to 10 minutes at 37°. Guanidine hydrochloride (2 \( M \), pH 7.2) was also ineffective for up to 8 minutes at 37°. Treatment for 30 minutes in 7.2 \( M \) urea at 0° gave no inactivation, but when this was followed by dilution into 7.2 \( M \) urea at 37°, an inactivation curve identical to that observed without pretreatment was obtained, except that the shoulder was shortened by 1 minute (Fig. 1).

Virus in 7.2 \( M \) urea (pH 7) very rapidly chilled after 5, 6, and 7 minutes in urea and dialyzed overnight against PBS, or diluted slowly (i.e., in 2½ hours) by dropwise addition of 20 volumes of PBS, gave inactivations identical with parallel samples in which the urea was abruptly diluted. Thus the inactivation was not due to osmotic shock to a structure perhaps weakened by hydrogen-bond disruption.

The reaction was temperature and concentration dependent. Up to 20 minutes at 18° and 30 minutes at 0° in 7.2 \( M \) urea (pH 8) gave no inactivation, and at 30° and pH 7 there was no inactivation after 8 minutes, 20% inactivation after 13 minutes, and 55% after 16 minutes. Table 1 shows the effect of varying the urea concentration and indicates that a fivefold dilution markedly slows virus inactivation. Thus the routinely used tenfold dilution, together with simultaneous chilling to 2°, is probably adequate to halt virus inactivation by 7.2 \( M \) urea.

Stark et al. (1960) report that the cyanate present at up to 0.02 \( M \) in 8 \( M \) urea solutions can react slowly with amino acids and proteins. The urea solutions, used by

<table>
<thead>
<tr>
<th>Urea concentration (M)</th>
<th>Residual virus after 8 minutes treatment (PFU/ml)</th>
<th>Residual virus after 65 minutes treatment (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.9 ( \times 10^5 )</td>
<td>5.7 ( \times 10^4 )</td>
</tr>
<tr>
<td>0.9</td>
<td>4.4 ( \times 10^4 )</td>
<td>3.5 ( \times 10^4 )</td>
</tr>
<tr>
<td>1.8</td>
<td>2.5 ( \times 10^3 )</td>
<td>1.0 ( \times 10^3 )</td>
</tr>
<tr>
<td>3.6</td>
<td>6.3 ( \times 10^2 )</td>
<td>&lt;10</td>
</tr>
<tr>
<td>5.4</td>
<td>20</td>
<td>—</td>
</tr>
<tr>
<td>7.2</td>
<td>&lt;5</td>
<td>—</td>
</tr>
</tbody>
</table>

* Virus at 37° was diluted into urea solutions at 37° to give 5 \( \times 10^3 \) PFU/ml and the indicated concentrations of urea, and at 8 minutes and 65 minutes samples were diluted tenfold into ice-cold PBS for assay.
the writer before the above report appeared, certainly contained some cyanate, as they were autoclaved and stored for as much as 2 months. The following experiments were accordingly done to see whether cyanate accounted for any of the observed effects of urea on poliovirus.

An 8 M urea solution, freshly prepared without heating or sterilizing, was adjusted to pH 2.3 with 10 N HCl and allowed to stand for 1 hour at 20° to decompose cyanate. The pH was readjusted to 7.1 with concentrated NaOH solution and the solution was chilled to 4°. Urea inactivation experiments were carried out forthwith at a final urea concentration of 7.2 M and at 37°, using this purified urea solution either alone or with sodium cyanate solution (pH 7) added to 0.02 M. The resulting two inactivation curves up to 10 minutes in urea were indistinguishable; both were identical with those done at pH 7.0 and shown in Fig. 1. Sodium cyanate alone, in PBS at pH 7.2, had no effect on the infectivity of poliovirus for up to 1 hour at 37°, in concentrations up to 0.2 M.

Infective RNA was prepared from a purified virus preparation (10⁶ PFU/ml in 0.02 M phosphate, pH 7) by three successive phenol extractions and one ether extraction. Samples of the resultant RNA solution were kept either for 30 minutes at 4° before assay or incubated for 8 minutes at 37° and then chilled to 4° for 20 minutes before assay; parallel samples containing 0.02 M sodium cyanate (pH 7) were treated identically. The infectivities of all samples after these treatments were indistinguishable by plaque assay (3 × 10⁶ PFU/ml). Thus cyanate had no effect on the rate of inactivation of poliovirus in urea, or on the stability of intact virus or of infective RNA. The unimportance of cyanate in urea inactivation of poliovirus was supported by the lack of protective effect of cysteine, thioglycolate, and ascorbic acid on urea inactivation (see below), despite the protective effect of GSH.

It is concluded that the inactivation of poliovirus in urea solutions is most likely the result of the expected mechanism of hydrogen bond disruption rather than of the alternatives examined above. The inactivation is independent of osmotic shock, is not due to thermal inactivation alone, and does not appear to be related to the cyanate in urea solutions.

Reversibility in PBS at 37° of most of the damage incurred in urea at 37°. A virus preparation was incubated for 5 minutes in 7.2 M urea at pH 7 and 37°, and at this time samples were diluted tenfold both into a tube of PBS kept at 37° and into a tube of PBS kept at 2°. Subsequent assay of the dilution at 2° compared with the untreated preparation indicated that 25% of the PFU survived this first urea treatment. The tube kept at 37° was sampled at times varying between 1 and 60 minutes, the samples being diluted tenfold into fresh 7.2 M urea at 37° and pH 7. After precisely 3 minutes, a portion of each of these second urea treatment mixtures was finally diluted tenfold into PBS at 2° for assay of infectivity. It was found that in all cases (i.e., after all times in PBS at 37° between urea treatments) the infectivity was halved during the second urea treatment. The ultimate survivors were thus always 12.5% of the original preparation; since these survivors were very many more than those to be expected (<0.1%) from the total of 8 minutes spent in 7.2 M urea at 37°, it is concluded that much of the hydrogen bond disruption in 7.2 M urea is repaired within 1 minute at 37° in absence of urea. However, since the second 3-minute urea treatment had no effect on untreated virus but reduced the infectivity of samples pretreated with urea by 50%, some of this damage is irreversible in at least half of the particles.

The Infective Product

Although it is fully infective, the 4-minute material must be expected to differ from the starting material because it succumbs to urea inactivation with little initial delay. The properties of the 4- and 5-minute products were accordingly compared with those of untreated virus.

Figure 2 compares the stability at 48° of the 2-, 4-, and 5-minute products of 7.2 M urea (pH 8) treatment with control virus, and shows that the products of urea treatments for these times were somewhat less stable than the original virion, but curi-
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ously, were not markedly different from one another. Figure 3 shows that the 4.5-minute urea product (7.2 M, pH 8) adsorbed to ERK cells at 0° at about half the rate of the untreated virus; this product might have contained a mixture of particles, some of which still adsorbed as rapidly as the original. Figure 4 shows that the infectivity of the untreated virus and all products up to 5 minutes were neutralized at the same rate by antibody.

Urea treatment for up to 5 minutes at pH 7 did not significantly affect the proportion of the surviving infectivity (about 70%) which sedimented from 5 ml of PBS in 50 minutes at 40,000 rpm in the Spinco SW/39 swing-out head. Like untreated virus, the infectivities of the 3- and 5-minute urea products (7.2 M, pH 8) were completely resistant to 2 mg sodium deoxycholate per milliliter, 5 cycles of freezing and thawing in PBS, 10 μg pancreatic ribonuclease per milliliter (16 minutes at 37°), 2.5 mg crystalline trypsin plus 2 mg Versene per milliliter, heterotypic poliovirus (type 3) antisemum, and dilution in distilled water, but unlike the untreated virus were inactivated to 10^−3 survivors in 1 week at 0° in PBS (untreated virus was completely stable).

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**Fig. 2.** Rate of loss of poliovirus infectivity (PFU) at 48°, virus being untreated (○), or pretreated for 2 minutes (△), 4 minutes (▲), or 5 minutes (●) with 7.2 M urea (pH 8, 37°). Virus was diluted tenfold into cold PBS after 2, 4, or 5 minutes in concentrated urea at 37°, and diluted tenfold again into PBS at 48° for inactivation. Samples from the four suspensions at 48° were further diluted tenfold into cold PBS at intervals for assay of surviving virus.

**Fig. 3.** Rate of poliovirus adsorption by ERK cells, virus being untreated (○) or pretreated for 4.5 minutes with 7.2 M urea at pH 8 and 37° (△). Cells, grown and maintained in suspension, were washed twice in PBS, and rocked at 5 X 10^7/ml and 0° in an ice bath with virus in PBS, samples of cells and virus being diluted into PBS at intervals and centrifuged for 5 minutes at 1000 rpm for assay of supernatant infectivity (PFU).

**Fig. 4.** Rate of loss of poliovirus infectivity (PFU) in presence of type-specific antiserum, virus being untreated (○) or pretreated for 2 minutes (●), 4 minutes (×) or 5 minutes (△) with 7.2 M urea (pH 8, 37°). Virus was diluted tenfold from warm concentrated urea into cold PBS at 2, 4, or 5 minutes, and diluted tenfold again into PBS at 37° containing antiserum (1:1000), samples being further diluted fiftyfold into cold PBS for assay of surviving virus.
In experiments undertaken to determine the density of the 4.4 minute product, this product was centrifuged with CsCl solutions as described in Fig. 5 so as to concentrate the product as a distinct band of definable density in an equilibrium density gradient. Figure 5 shows that most of the P32 of the 4.4-minute product had the same density as had the original virus, and so the bulk of the particles were unchanged in density. The distribution of infectivity was not investigated, but since little had been lost it was expected to reside with the bulk of the P32.

It is therefore concluded that the virus infectivity still fully present by 4 minutes (and probably the infectivity still surviving at 5 minutes) are properties of particles essentially similar to the original virion. In particular they possess in a functionally intact state the critical site blocked by neutralizing antibody. There is, however, some derangement which is presumably in the protein coat, resulting in partial impairment of adsorption and change in thermostability.

**The Noninfective Product**

The cesium chloride density gradient experiment of Fig. 5 indicates an increase in density of a small portion of the P32-containing material in the 4.4-minute (7.2 M, pH 8) product. A similar experiment using a higher CsCl density and that proportion of the 6.25-minute product which sedimented easily (Fig. 6) showed an almost complete change to a material very much more dense than the virus which did not form a distinct band; a considerable amount of the P32 did not sediment, however. Gel filtration through a Sephadex column (Fig. 7) showed the bulk of this material to be of high molecular weight, but the molecular weight decreased markedly on incubation alone or with 10 μg/ml ribonuclease.

**Quantitative small-volume centrifugation.**

A simple method was employed for centrifugation analysis of small volumes which allowed quantitative comparisons of the sedimentation rates of intact virus and RNA phenol-extracted from P32-labeled virus. Identical aliquots of virus, virus-urea product, or RNA (2 ml) were centrifuged...
Fig. 7. Gel filtration at 0° of P3² from labeled poliovirus through columns of Sephadex G-75 (lower limit for complete exclusion given as molecular weight 40,000-50,000). A. Virus untreated or incubated with ribonuclease (fractions marked with black circles contain hemoglobin added as a marker). B. Virus treated with 7.2 M urea (pH 7, 37°) for 6 minutes (fractions marked with black circles contain urea). C. Virus treated with 7.2 M urea for 6 minutes and incubated for 30 minutes at 37° with or without 10 μg/ml ribonuclease (fractions marked with black circles contain urea). The positions of the urea and hemoglobin were noted visually, the former as a translucent band, the latter as a red color.

in a Spinco model L No. 40 angle rotor at 35,000 rpm, under 10 ml of heptane and over a small (0.15 ml) pellet of 2.5% agar containing a wisp of absorbent cotton to strengthen it; this pellet effectively prevented any resuspension of completely sedimented material. Centrifugation was interrupted at the intervals indicated in Figs. 8 and 9, when the aqueous supernatant of one tube was removed by Pasteur pipette for assay of radioactivity or infectivity; the recovery of material treated identically, except that centrifugation was omitted, was quantitative. In order to eliminate self-absorption during assay of radioactivity in urea-containing preparations, they were

Fig. 9. Rate of sedimentation of radioactivity from poliovirus labeled in RNA with P3² (batch PIG) or in protein with S⁸ methionine and cystine (batch SIB). The method of centrifugation and sampling is described in the legend of Fig. 8. PIG after 8 minutes in 7.2 M urea at 37°, A; RNA extracted from PIG with phenol, O; RNA extracted from PIG after 8 minutes in 7.2 M urea at 37°, •. The dashed line is taken from Fig. 8 and is included for comparison; it is poliovirus labeled in protein (SIB) and treated with urea.
Fig. 10. Correlation between rate of inactivation of infectivity (dashed line from Fig. 1) and solubilization of P$^{32}$ (○, batch PIG) and S$^{35}$ (●, batch SIB) from labeled virus in 7.2 M urea (pH 7, 37°). Labeled virus was diluted into cold PBS from concentrated urea at various times. The arrows on two curves indicate the points of 50% of maximum effect. Solubilization was measured as that radioactivity not sedimented after 120 minutes at 35,000 rpm in a 2-ml volume under heptane in the Spinco model L 40 rotor.

first treated in polythene planchettes with 1 mg of crystalline urease for 3 days at 37° to reduce the total solid matter to 3 mg/cm$^2$.

By this method (Figs. 8 and 9) the 50% sedimentation times of virus and RNA, respectively, were found to be 8 and 62 minutes, which are consistent with the sedimentation constants of 156s and 37s (Holland et al., 1960b).

Figures 8 and 9 show that a portion of the P$^{32}$ from the 8-minute (7.2 M, pH 7) urea product sedimented almost as rapidly as intact virus. In a total of seven replicate experiments this portion varied from 25 to 45% of the total P$^{32}$, (using two preparations of labeled virus) and Figs. 8 (●) and 9 (△) show two such replicate experiments. However, the unsedimented residue was only slightly or not at all sedimentable even for times which sedimented free RNA (Fig. 9).

Extraction of the urea-treated labeled virus with cold phenol gave infective RNA preparations of which about half of the P$^{32}$ sedimented at 35,000 rpm and the residue did not sediment (Fig. 9). Considering only the sedimentable portion, 50% was sedimented in 75 minutes, a length of time of the order of the 62 minutes taken for 50% sedimentation of the P$^{32}$ phenol-extracted from untreated virus. The ratio of the total infectivity (when assayed as PFU of infective RNA) in the RNA extracted from the original labeled virus to the total infectivity in the RNA extracted from the urea product was about 2 to 1. However, the P$^{32}$ not precipitated at 10% trichloroacetic acid at 2° (measured before phenol extraction) was negligible in both control and urea-treated preparations (3.5% and 5.2%, respectively), so that the inactivated RNA was only partially degraded. The proportion of the urea-treated virus which sedimented like untreated virus was not significantly affected by heat inactivation before urea treatment (for up to 17 minutes at 48°, giving about 10$^{-2}$ survivors), a result indicating that the nondisrupted fraction was not simply a proportion of the original virus which had been thermally inactivated.

Treatment with urea was found to result in a solubilization of RNA and protein, which in general increased with time of treatment. When virus labeled in either the RNA or the protein was incubated for various times in 7.2 M urea, and the product was centrifuged for 120 minutes by the quantitative small-volume procedure, the solubilization of the RNA and some protein was found to be abrupt (Fig. 10). The time for solubilization of 50% of that RNA which ultimately became soluble coincided with the inactivation of 50% of the PFU. The protein release curves also possessed a point of inflexion of these times. It will be noted that at all times, but especially between 4 and 8 minutes, there was a marked separation between protein, which mostly sedimented, and RNA, which mostly did not. Allowing for the 15% or so rendered soluble (Fig. 8), that virus protein which sedimented can be seen to do so only a little more slowly than did intact virus. The solubilization of P$^{32}$ in urea was complete by 8 minutes in one experiment, but that of the protein continued up to 20 minutes. In other experiments designed to test the com-
RUPTURE OF POLIOVIRUS BY UREA

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pleteness of the reaction, further incubation up to 120 minutes did not solubilize significantly more S3 5 or P3 2.

It is therefore concluded that 7.2 M urea causes an abrupt rupture of the bulk (55-75%) of the virus particles. This rupture is presumably sufficient to cause loss of infectivity and coincides with this effect. Rupture leads to the liberation of the phosphorus of the RNA in soluble form, and also solubilizes a small portion of the protein; the bulk of the protein remains sedimentable at a rate similar to that of virus and is presumably a shell. Figure 7 has shown that the phosphorus liberated after 6 minutes in urea remains in the form of ribonuclease-sensitive high molecular weight polynucleotides. Since the polynucleotides have become nonsedimentable after 8 minutes in urea (Fig. 9), they have become less highly polymerized than the intact RNA from the original particles and are probably almost entirely noninfective; their degradation may well be due to traces of ribonuclease still present in some batches of purified virus. This unruptured virus is not infective, but it is not known whether urea caused its inactivation or whether it was noninfective before the experiment began.

The Variable Residual Infectivity

It was mentioned in the section describing the inactivation curve that, while the inactivation by urea after 5 minutes was usually exponential to 10⁻⁷ survivors (using the agar cell-suspension assay), there was often a small resistant fraction. This fraction was generally apparent after 8 minutes in urea, but the amount varied from experiment to experiment. The following experiments were done to determine the nature of this fraction, using the 8-minute urea product.

Experiment 1: Some attempts to obtain plaques from the 8-minute urea product on monolayers were successful. In these experiments, the plaque counts in monolayer assays, obtained when the urea product was diluted in M NaCl or in 0.8 M Na₂SO₄ (pH 8), were five- to tenfold greater than when it was diluted in parallel in PBS, whereas untreated virus and the 5-minute urea product reproducibly gave the same plaque count with all diluents. Thus the residual infectivity could be increased by procedures which increased the infectivity of RNA but not of intact virus.

Experiment 2: Pancreatic RNase markedly decreased the plaque count of the residual infectivity when prepared in the presence (Table 2) or, in other experiments, in the absence of GSH (the effect of GSH is

<table>
<thead>
<tr>
<th>Material</th>
<th>Dilutions plated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻¹</td>
</tr>
<tr>
<td>Poliovirus treated for 10 minutes with urea</td>
<td>50, 25, 12, 4, 5, 2, 1, 0, 8</td>
</tr>
<tr>
<td>Urea treatment followed by ribonuclease</td>
<td>4, 5, 5, 9, 0, 0, 1, 3, 0, 0, 1, 2</td>
</tr>
</tbody>
</table>

* 10 µg/ml, 15 minutes at room temperature.

After tenfold dilution into cold PBS, the material was plated, as for infective RNA, in 0.8 M Na₂SO₄ at pH 8, on monolayers formed in 20% calf serum medium. Values = plaque count.

TABLE 3

The Change in the Plating Properties of Poliovirus Infectivity during Treatment of Virus with 7.2 M Urea (pH 7) at 37° in Presence of Reduced Glutathione (10⁻² M)*

<table>
<thead>
<tr>
<th>Minutes in urea</th>
<th>Dilutions plated</th>
<th>Un-diluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>19</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>51</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>33</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

* Assayed by the agar cell-suspension method. Values are plaque counts.
COOPER discussed more fully below). Thus the RNA of this residual infectivity is accessible to RNase.

Experiment 3: The plaque count of the residual infectivity was unchanged by centrifugation for 1 hour at 35,000 rpm, using the quantitative small-volume procedure described above. The infectivity of intact virus, treated in parallel, was completely sedimented. Therefore the infectivity is in a unit of smaller molecular weight or much less dense than the original virus; the second alternative does not apply since purified virus preparations were used, and the only interpretation remaining is that the infectivity resides in RNA in soluble form.

It is therefore concluded that urea inactivation of poliovirus liberates some soluble RNA in an infective form susceptible to RNase. This may be free RNA or ribonucleoprotein. The interpretation of the release of soluble RNA is consistent with: (1) the frequent lack of proportionality between concentration of the residual fraction and plaque count (Table 3); and (2) its extraction efficiency in $10^{-2}$ M GSH being generally of a similar order to that of RNA extracted by phenol.

Since poliovirus RNA is very sensitive to RNase—$10^{-4}$ µg RNase per milliliter gives a half-life of infectivity of <2 minutes at 28° (Holland et al., 1960b)—traces of RNase present in the purified preparations are likely to have caused the slight degradation and considerable inactivation of the RNA described in preceding sections. Urea as a protein-denaturing agent is much less efficient than phenol, and is likely to be similarly less efficient in preventing or removing RNase activity.

Addition of GSH ($10^{-2}$ M) to the urea during inactivation appeared to make the residual infectivity greater (Figs. 1 and 11) and more reproducible, although it was still sensitive to pancreatic RNase (Table 2). The length of the shoulder of the inactivation curve was not much affected by GSH. Reducing agents, other than GSH, at $10^{-2}$ M and pH 7.6 (cysteine, thioglycolate, and ascorbic acid) and oxidized glutathione, did not protect the residual infectivity from inactivation (Fig. 11). An independent experiment showed that GSH ($10^{-2}$ M) did not affect the inactivation of poliovirus infective RNA by 10 µg/ml pancreatic RNase, although other species of RNase, sensitive to GSH, may well be present in the virus preparations.

**DISCUSSION**

High concentrations of urea at 37° appear to cause a progressive damage to poliovirus, presumably by the disruption of hydrogen bonds; most but not all of the damage is reversible during the early stages of degradation. Certain functions of the protein coat are slightly disturbed, notably adsorption to cells and resistance to thermal inactivation, but the site on the virus affected by neutralizing antibody is not noticeably altered.

At a certain critical and reproducible stage the urea treatment causes most particles to

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**Fig. 11.** Effect of reducing agents (all at $10^{-4}$ M) on rate of loss of poliovirus infectivity (PFU) in 6.4 M urea (pH 7.6 in 0.1 M Tris buffer) at 37°. Control (in Tris buffer but no reducing agent), O; reduced glutathione, •; cysteine, △; oxidized glutathione, thioglycolate or ascorbate, ▲.
rupture suddenly and thereby become non-infective, liberating the RNA in a soluble form susceptible to nucleases. Some of the virus preparations contain traces of these nucleases, but on occasion some of the liberated RNA has retained its infectivity, particularly in the presence of reduced glutathione. This indicates that urea has liberated the RNA intact from at least some of the particles.

Most of the protein shell does not become soluble and sediments at a speed similar to that of intact virus; the small proportion of the protein which does become soluble is released at the same time as the RNA and may represent a protein different from that of intact virus; the small proportion of the particle which does become soluble is released at the same time as the RNA and is necessarily involved during the infection of a cell by the virus. It is, however, quite feasible that the cell surface, in presenting a macromolecular pattern complementary to that of a site on the virus surface, thereby breaks a number of hydrogen bonds and thus causes the particle to release its nucleic acid suddenly in the direction of the cell.

Other experiments similar to the above (Cooper, unpublished) have indicated that moderate heat treatment can also liberate the nucleic acid of poliovirus, although heat treatment for shorter times can destroy the virus infectivity without affecting adsorption or the contained infective RNA, and without disrupting the virus. It may be that the mechanism which releases the RNA is particularly sensitive to hydrogen bond disruption, and is thus activated by both urea and heat treatment.

ACKNOWLEDGMENTS

I am indebted to Dr. Michael Fenwick for preparing the purified isotope-labeled virus. The able technical assistance of Mr. H. Cumming and Miss J. Constable is gratefully acknowledged.

REFERENCES


The Effect of Concentrated Urea Solutions on Poliovirus Strains Adapted to Different Growth Temperatures

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This paper compares the effect of concentrated urea solutions on several poliovirus strains adapted to grow at widely different temperatures. The curves relating survival of infectivity to time spent in urea all have the shape characteristic of a multistep reaction. Strains increasingly tolerant to high growth temperatures are increasingly stable in urea, yielding curves with longer "shoulders" and generally less steep subsequent slopes. Hence these strains show an association between sensitivity to urea and sensitivity to high growth temperatures. However, when urea-resistant variants are selected from a urea (and temperature) sensitive stock by treatment with $7.2 \, M$ urea, it is found in this case that urea resistance is accompanied by only a very small increase in the ability to grow at higher temperatures.

It is concluded that the adaptation of poliovirus to a new optimal growth temperature selects for at least two independent genetic characters. One character has a very large effect on the thermal growth requirement, whereas the other predominantly controls the strength of certain labile bonds in the virion itself, probably hydrogen bonds in the capsid.

INTRODUCTION

Strains of poliovirus having different optimal growth temperatures may readily be developed from a single parental stock (Dubes and Wenner, 1957; Lwoff, 1959). Lwoff and Lwoff (1961a–c; 1962) investigated the growth of such strains in the presence of D$_2$O and dilute urea solutions, and at various temperatures. These treatments modify the strength of hydrogen and hydrophobic bonds, and the strains behaved as though an increase in their optimal growth temperature was linked with or resulted from a greater cohesion between the molecules involved in certain viral growth processes.

Concentrated urea solutions progressively damage the poliovirus particle, probably by simple rupture of such cohesions (Cooper, 1962). The damage by urea culminates in loss of infectivity with simultaneous solution of the RNA, while the protein remains sedimentable. Thus the rate of inactivation of a poliovirus strain by urea should give a measure of the cohesive forces in individual virions of that strain.

This paper compares the cohesive forces in virions of poliovirus strains adapted to different optimal growth temperatures by comparing their inactivation kinetics in $7.2 \, M$ urea. The purpose of this work was to see whether the adaptation of a strain to growth at a new optimum temperature also causes a change in the cohesive forces of the virions themselves. The results show that such a change in the nature of the virion does indeed occur during temperature adaptation, but that the observed characters of urea sensitivity and of optimal growth temperature are not necessarily covariant.

METHODS

Virus assays employed the bicarbonate-free agar cell-suspension method (Cooper,
plaque assays and to grow virus stocks.

Virus strains were all poliovirus type 1. Strains Brunenders (BE), the Sabin attenuated LSc2ab (VS), and Brunhilde were adapted to ERK cells and were twice plaque purified; stocks were prepared by the bulk growth procedure described below. Strains VS.25 (4), VS.27 (5), VS.35 (13), and VS.41 (11) were obtained from Dr. A. Lwoff (Institut Pasteur, Paris), who had derived them from VS as designated by the numbers (e.g., for VS.25 (4), by 4 passages at 25°). These strains had accordingly been extensively adapted to their new growth temperature. Stocks of the Lwoff strains were prepared, without plaque purification in order not to change their character, by two passages in ERK cells at 36° (31° for VS.25 and VS.27), followed by concentration as described below. BE stocks containing deuterium were prepared as follows. Five plaque-forming units (PFU) per cell were added to washed monolayers; after 1 hour at 20° medium was added comprising 1 volume of twofold supplemented Earle's saline as used for plaque assays (Cooper, 1961) plus 1 volume of 99.83% D₂O. Cytopathic effect was extensive after 16 hours at 36°, when the cultures were frozen and thawed, and used as a virus stock without subsequent treatment.

**Standard bulk virus growth procedure.** A suspension of 10⁷ ERK cells per milliliter in a serum-free version of medium CSV.6 was infected by adding 3-5 PFU of poliovirus per cell. The suspension (100-400 ml, with a depth of up to 10 cm) was stirred for 16 hours at 35° and gassed with 5% CO₂ in oxygen; then the whole culture was frozen and thawed. Yields were 100-300 PFU/cell with oxygen/CO₂; air/CO₂ generally gave much poorer yields, presumably because the dense cell suspensions were anaerobic. The preparation was then centrifuged for 15 minutes at 10,000 rpm in a Spincno no. 30 rotor, and the supernatant was recenterfuged for 180 minutes at 30,000 rpm. The resultant pellet was resuspended after standing overnight in 0.02 M phosphate (pH 7), giving approximately 10- to 30-fold concentration of infectivity. This material was clarified by low speed centrifugation if necessary.

**Determination of rt value.** The rt value of a strain expresses the upper range of its thermal growth requirement (Lwoff and Lwoff, 1961a), and equals the supraoptimal temperature at which the yield is reduced to 10% of the optimal yield. For each strain the rt value was determined directly on the stocks used for urea inactivation. To determine the rt value, tubes containing a monolayer of 8 × 10⁴ ERK cells were infected at 20° with 0.1 ml of medium CSV.6 containing about 10⁵ PFU of virus. After 1 hour, each tube received 1 ml of CSV.6, then was incubated for 8 hours in one of a series of water baths set at temperatures between 34° and 40°. The cultures were then frozen and thawed, and assayed for infectivity. The rt value was estimated graphically as that supraoptimal temperature at which the yield was 10% of the optimal yield. The rt values (average of several experiments) were: VS.25, 37.0°; VS.27, 37.6°; VS, 38.1°; VS.35, 38.3°; BE, 39.0°; Brunhilde, 39.3°; VS.41, 40.5°; all these values are subject to an uncertainty of ±0.2° and are close to those found by Lwoff and Lwoff (personal communication). Most experiments were performed with strains VS.25, VS, BE, and VS.41. In preliminary experiments, it was found that VS.27 was inactivated in urea very similarly to VS.25, and Brunhilde similarly to BE, whereas VS.35 was intermediate in rate of inactivation between VS and BE. Further experiments with strains VS.27, VS.35, and Brunhilde were not performed.

**Urea treatment.** The procedure described by Cooper (1962) was followed, in which 0.2 ml of a warm virus preparation was added at zero time with rapid mixing to a tube containing 1.8 ml of urea solution (8 M, or otherwise as indicated), at pH 7.0, maintained in a water bath at the temperatures indicated in the text. A sample of 0.2 ml was taken within 15 seconds and at once diluted 10-fold into cold phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) to stop the reaction. Further samples were
taken at intervals, and all were assayed for infectivity shortly afterwards. The urea solution was freshly made up for most experiments, adjusted to pH 7.0 with 0.01 M HCl, and stored at 4° for not more than 2 weeks; it was used without sterilization. These precautions were taken to minimize cyanate contamination of the urea (Stark et al., 1960). The urea was dissolved directly in deuterium oxide (99.83% w/w) where appropriate, and treated in the same fashion as the normal urea solutions; the final strength of D$_2$O was 90% in the mixtures of deuterated urea solution and virus.

RESULTS

A Comparison of the Stabilities in 7.2 M Urea of the Infectivity of Poliovirus Strains of Different rt Values

The curve relating the surviving infectivity of poliovirus type 1 (strain BE) to time spent in concentrated urea solutions has the shape characteristic of a multistep reaction (Cooper, 1962). The infectivity is relatively unaffected for 4–5 minutes, then is rapidly lost. Figure 1 shows that, despite considerable differences in their rt values, the various type 1 poliovirus strains were all inactivated by 7.2 M urea (pH 7.0, 37°) in a similar fashion. However, strains of lower rt value were less stable in urea than were those of higher rt value. Strains of progressively lower rt produced curves with progressively shorter "shoulders" and steeper subsequent slopes.

The Effect of Temperature on the Stability of Poliovirus in 7.2 M Urea

Small differences in the temperature of urea treatment (7.2 M, pH 7.0) of strain BE produced a family of inactivation curves (Fig. 2) very similar to the family of curves produced by urea treatment of strains of different rt value (see Fig. 1). Differences in temperature of 2° produce changes in the inactivation curves (Fig. 2)

![Graph](image_url)

**Fig. 1.** The effect of 7.2 M urea (pH 7.0, 37°) on the survival of infectivity of four poliovirus strains of different rt value. The strains, with rt values in parentheses, were VS.25 (37.0°), VS (38.1°), Brunenders (BE, 39.0°), and VS.41 (40.5°). Virus was added to urea solutions at zero time, and samples were taken initially and after the indicated times in urea, as described in the Methods, for assay of surviving infectivity.
which resemble those changes produced by differences in \( rt \) value of 1–2° (Fig. 1).

In another type of experiment, the stabilities of VS, BE, and VS.41 in 7.2 \( M \) urea (pH 7.0) were compared at various temperatures between 30° and 40°: samples were taken after treatment with urea at these temperatures for fixed times of either 6 or 9 minutes. This experiment was expected (from the multistep nature of the urea inactivation curve, Fig. 1) to show little inactivation up to a certain temperature, above which the loss of infectivity in the fixed time would increase markedly. The results are shown in Fig. 3, and are similar to those expected. A critical temperature cannot be determined precisely, but strains of higher \( rt \) value were again more stable in urea. An increase in \( rt \) value of 1–2° and a decrease in temperature of urea treatment of 1–2° produced similar effects on the inactivation curves.

The Effect of Urea Concentration on the Stability of Poliovirus in Urea Solutions

Small decreases in urea concentration produced a family of inactivation curves of strain BE (Fig. 4) which resembled both the family of curves produced by urea treatment of the different strains and the family of curves produced from strain BE by varying the temperature of treatment.

The Effect of D\(_2\)O on the Stability of Poliovirus in 7.2 \( M \) Urea

Deuterium generally forms stronger bonds with other atoms than does hydrogen (Wiberg, 1955), and, as expected, certain hydrogen bonds are strengthened by the substitution of deuterium (Plourde, 1961). Accordingly poliovirus was treated with urea in presence of D\(_2\)O in order to study the effect of strengthening the hydrogen bonds in the system. Three methods were used: (a) urea was made up in D\(_2\)O (strain
Fig. 3. The effect of temperature on the survival in 7.2 M urea (pH 7.0) of the infectivity of three poliovirus strains of different rt value. The strains, with rt values in parentheses, were VS (38.1°), Brunenders (BE, 39.0°), and VS.41 (40.5°). Virus was added to urea solutions maintained in water baths at the indicated temperatures, and samples were taken initially and after 6 minutes' (left-hand group of curves) and 9 minutes' (right-hand group of curves) treatment with urea, as described in the Methods, for assay of surviving infectivity.

BE being treated with a solution of urea containing 90% by volume of D₂O; (b) BE was grown in media containing 50% D₂O by volume; and (c) methods (a) and (b) were combined. Whichever of the three methods was used, the presence of D₂O increased the stability of BE in 7.2 M urea (Fig. 5), showing that D₂O counteracted the effects of urea. A similar counteraction between D₂O and urea has been shown in the growth of poliovirus (Lwoff and Lwoff, 1962). Figure 5 shows that increasing the proportion of deuterium in the system resulted in a family of curves similar to those produced by (a) increasing the rt value, (b) decreasing the temperature, and (c) decreasing the urea concentration.

Poliovirus grown in media containing D₂O was inactivated more slowly in 7.2 M urea than was poliovirus grown in normal media (Fig. 5). This was true whether the two preparations of virus were compared in H₂O urea or in 90% D₂O urea solutions. Thus inactivation by urea seems to depend critically (but perhaps not solely) upon the rupture of hydrogen (or deuterium) bonds formed during virus synthesis, an appreciable proportion of which are not accessible to free proton exchange because they are contained within the virion.

The Separation of the Characters of Urea Resistance and of Ability to Grow at a High Temperature

Presumably the adaptation of a strain to a new optimal temperature results from the selection of mutants which grow better at that temperature. In the adapted poliovirus strains used here, the urea resistance is shown above to increase or to decrease with the rt value. This association may reflect either a single mutation which has affected both characters, or multiple mutations affecting two independent characters, which in their new expression are both selected because they each afford a growth
Fig. 4. The effect of urea concentration on the survival of infectivity of poliovirus strain Brunenders in urea solutions at 37°C and pH 7.3. Portions of a warm virus preparation were added at zero time to urea solutions maintained in a water bath, such that the final concentrations of urea were 7.2 M, 6.4 M, 5.6 M and 4.8 M. Samples of the urea-virus mixtures were taken initially and after the indicated times in urea, as described in the Methods, for assay of surviving infectivity.

advantage under the new conditions. If the two characters are independent, it should be possible to separate them.

In order to attempt this separation, urea-resistant variants were selected by two serial urea treatments of a parental stock of strain VS (which has a low rt value and a low resistance to urea) in the following way. A portion of this stock was inactivated with 7.2 M urea (7 minutes at 37°C) to 10^-4 to 10^-5 survivors. The reaction was stopped by diluting 100-fold into cold PBS, and 5 ml of this dilution, containing 10-100 PFU/ml, were added to monolayers of 2 x 10^7 ERK cells from which the medium had been removed. After 1 hour at 36°C, the monolayers were washed twice, overlaid with 80 ml of medium CSV.6, and incubated until cytopathic effect was extensive (2-3 days at 36°C). The cultures were then frozen and thawed and clarified at 10,000 rpm in the Spincio model L; the virus was concentrated 50-fold by sedimentation at 40,000 rpm. This concentrated stock was termed VS.U1; a second concentrate (VS.U2) was prepared in the same manner by urea treatment of VS.U1. As a control, some of the same parental VS stock was maintained in PBS for 7 minutes at 37°C, then diluted 10^3-fold into cold PBS containing 0.72 M urea. This dilution was closely similar in composition and infectivity to the dilution which was used as inoculum to prepare VS.U1, and was used in a similar manner to prepare a first control concentrate VS.C1. A second control concentrate (VS.C2) was prepared from VS.C1 in the same way. Comparable control and urea-treated stocks were prepared in parallel, and care was taken to ensure that their handling was closely similar.

This method of enrichment selection was used for two reasons. Firstly, any urea resistant variant was more likely to be
selected than if individual survivor plaques had been picked. Secondly, some information on the proportion of urea-resistant variants in the surviving population can be inferred from the urea inactivation curves of the derived strains. As can be seen from the high urea resistance of VS.U1 and VS.U2 (Fig. 6), this proportion is likely to be very large.

Figure 6 compares the urea sensitivities of the derived stocks with those of VS and VS.41. Stock VS.C2 was as sensitive to urea treatment as was the parental strain VS, but VS.U1 and VS.U2 were as resistant to urea as was VS.41.

Thus urea-resistant strains were very readily selected from strain VS. That this selection was due to treatment with 7.2 M urea, not to subsequent handling, is shown by the urea sensitivity of VS.C2, which was prepared exactly as was VS.U2 except that treatment with 7.2 M urea at 37° was omitted.

The $r_t$ values of VS.C2, VS.U1, and VS.U2 were not determined directly, but their efficiencies of plating at 40° are compared in Table 1. Stocks VS.C2 and VS were both completely prevented from forming plaques at 40°, and VS.U1 and VS.U2 were also very sensitive to high temperatures compared with VS.41. But VS.U1 and VS.U2 were able to produce some plaques at 40°, whereas VS and VS.C2 were not.

Thus two stocks (VS.U1 and VS.U2) have been obtained by the selective action of urea. These strains are highly resistant to the action of urea and yet are strongly inhibited in growth at 40°. Hence the characters of urea resistance or sensitivity and ability to grow at high or low temperatures,
while apparently being selected together during adaptation to growth at a high or a low temperature, are nevertheless readily separable on selection for urea resistance. These characters are thus not necessarily covariant. This observation is discussed below.

DISCUSSION

The Nature of the Viral Bonding Broken by Urea

Concentrated urea solutions affect both hydrogen and hydrophobic bonds (Kauzmann, 1959). Poliovirus is probably inactivated in urea solutions by simple rupture of such labile inter- and intramolecular cohesions (Cooper, 1962), presumably in the viral capsid. As to whether hydrogen or hydrophobic bonds are the more important in the poliovirion, Mandel (1962) found that 5% solutions of sodium dodecyl sulfate do not significantly inactivate poliovirus at pH 7, neither do methanol and butanol (Schwerdt and Schaffer, 1956), nor 50% dioxane in water (Cooper, recent observation). If, as seems likely, hydrophobic bonds are very sensitive to these reagents, then it follows that at pH 7 poliovirus infectivity depends critically on its hydrogen bonds: either the hydrogen bonds alone may maintain the structure of the virion, or some intact hydrogen bonds prevent the rupture of certain hydrophobic bonds. That hydrogen bonds are indeed critical in urea inactivation (i.e., to some extent rate limiting) is indicated by the finding that the growth of virus in D₂O counteracts the
TABLE 1
COMPARISON OF THE ABILITY OF POLiovirus STRAINS TO PRODUCE PLAQUES AT 90° AND AT 40°

<table>
<thead>
<tr>
<th>Strain</th>
<th>Efficiency of plating at 40°</th>
<th>Expt. no. 1</th>
<th>Expt. no. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS</td>
<td>&lt;10^{-6}</td>
<td>&lt;10^{-7}</td>
<td></td>
</tr>
<tr>
<td>VS.C2</td>
<td>&lt;10^{-3}</td>
<td>&lt;10^{-6}</td>
<td></td>
</tr>
<tr>
<td>VS.U1</td>
<td>2.5 × 10^{-4}</td>
<td>5.5 × 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>VS.U2</td>
<td>10^{-3}</td>
<td>3 × 10^{-6}</td>
<td></td>
</tr>
<tr>
<td>VS.41</td>
<td>1.0</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

*Replicate dilutions of the various strains, plated on galactose agar as described by Cooper (1961), were incubated for 3 days, some replicates at 36° and some at 40°. The plates were then stained and plaques were counted. The efficiency of plating at 40° equals the ratio of the titre (PFU/ml) calculated from the 40° assay to the titre calculated from the 36° assay.

The end point was not obtainable; cells are killed by VS at 40° without producing virus, so that although low dilutions gave confluent cell lysis, no higher dilutions gave a plaque.

effect of urea. Such an effect can only reflect the rupture of critical deuterium bonds incorporated in the particle.

Whether the infectivity of the virion also depends critically on its hydrophobic bonds (either because they alone can maintain the structure or because they block the rupture of hydrogen bonds) is not yet clear. However, Mandel (1962) also found that poliovirus, although stable at pH 2, is rapidly inactivated by as little as 0.001% sodium dodecyl sulfate at that pH, and it may be that in acid solutions the hydrogen bonds are sufficiently weakened to enable the hydrophobic bonds to control the infectivity. Thus hydrogen and hydrophobic bonds may well be equally important in that they provide alternative means of keeping the virion intact.

The Nature of the Change in the Virions That Accompanies Adaptation to a New Growth Temperature

It has been presumed in this work that certain simple procedures will decrease the rate of rupture by urea of the hydrogen bonds in the poliovirion. These procedures are to decrease the urea concentration and the temperature, and to increase the amount of D_2O present. All procedures result in a particular change in the inactivation kinetics: the "shoulder" of the inactivation curve becomes longer, and the subsequent slope decreases. This change is of course predictable if certain assumptions are made.

However, the same type of change in urea inactivation kinetics also results from a change in the rt value of a strain by deliberate adaptation to a new optimal growth temperature. An increase in rt value leads to a decrease in the extent of urea inactivation, and vice versa. A range of small variations in all four parameters (urea concentration, temperature, D_2O content, and rt value) in fact leads to four similar families of inactivation curves. It is a fair inference, therefore, that the successful adaptation of a strain of poliovirus to, say, a higher growth temperature is accompanied by an increase in the strength and/or number of critical cohesions in individual virions comprising that strain. The finding that an increase in the temperature of urea treatment by 2° produces the same change in kinetics of inactivation as does a decrease in the rt value of 1–2° also supports this conclusion. It is likely that the critical cohesions exist in the capsid.

The Genetic Relation between rt Value and Urea Sensitivity

The following three facts show that the genetic character which controls the strength of the intraparticulate cohesions is distinct from that which controls the rt value. Firstly, and foremost, the characters in their broad expression are not necessarily covariant and can easily be separated (see above). Secondly, Lwoff et al. (1962) observed that the step or steps in the growth process which are inhibited at 40° occur about midway between penetration and maturation, which are the steps of virus growth in which the capsid and therefore probably the cohesive strength of the particles can be expected to be involved. Thirdly, these authors find that maturation
is not blocked by temperatures (e.g., 40°) that block the complete growth cycle.

Therefore, a simultaneous change both in $t$ value and in the strength of bonding in the particle on adaptation to a new growth temperature reflects two independent genetic responses to a certain selection pressure, rather than two expressions of a single genetic event.

As to how a new growth temperature might select virions of different cohesive strengths, it might be predicted that high growth temperatures will tend to eliminate weakly bonded progeny by hindering the closing of their capsids on maturation, whereas low temperatures will tend to eliminate strongly bonded virions by hindering the opening of their capsids on penetration. However, this prediction implies that some effect on the thermal sensitivity of the growth process is in fact to be expected from differences in strength of capsid bonding. Such an effect, although very small, may be found in the significant difference between the plating efficiencies of VS.U2 and of the parental stock VS. If so, it amounts to the presence of a very small degree of covariation between the characters of thermal growth requirement and urea sensitivity.

ACKNOWLEDGMENTS

I wish to thank Mr. Hamish Cumming and Miss Jennifer Constable for skilful and extensive technical assistance. I am also grateful to Dr. André Lwoff for his kindness in sending several strains of poliovirus and for valuable discussions.

REFERENCES


The Mutation of Poliovirus by 5-Fluorouracil

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Accepted October 15, 1963

Poliovirus grown in 4 mM 5-fluorouracil (5-FU) at an optimum temperature (36°) becomes more "heat defective"; that is, the ratio of plaques formed at supraoptimal temperatures to plaques formed at 36° ("plating efficiency") is decreased by a factor of 3-10. The plating efficiency at an infraoptimal temperature (30°) is unchanged. These findings are true for strains with very different plating efficiencies at both high and low temperatures. The enhanced heat defectiveness is retained after subculture in absence of 5-FU: since selection of preexisting heat-defective (hd) mutants is shown to be unlikely, it is concluded that 5-FU is highly mutagenic for poliovirus when judged by the character of plating efficiency at high temperatures. This is confirmed by the plaque isolation of hd mutants, which comprise at least 10% of the progeny of a nondefective (hd+) strain grown in 1 mM 5-FU; normal hd+ progeny contain 1-2% of hd mutants.

Poliovirus growth is inhibited by 5-FU to an extent which depends on the intrinsic heat defectiveness of the strain: the lower the plating efficiency at high temperatures, the poorer the growth in 5-FU at 36°.

INTRODUCTION

Certain purine and pyrimidine analogs readily replace a natural base in a nucleic acid if they are present during replication. In particular, 5-fluorouracil (5-FU) replaces uracil in RNA (Gordon and Stachelin, 1959; Horowitz and Chargaff, 1959) and may induce synthesis of an abnormal protein (Naono and Gros, 1960). The function of the RNA is not always detectably affected: much 5-FU can be built into the RNA of poliovirus (Munyon and Salzman, 1962) and tobacco mosaic virus (Holoubek, 1963) without ostensibly changing any of several properties of the viral coat proteins, such as their amino acid content. No mutation of these viruses was recorded.

Thus about 30% replacement of uracil by 5-FU appears not to affect the coding functions of uracil in the formation of certain virus proteins. Nevertheless 5-FU is to be expected to induce some miscoding in RNA viruses, because halogenated uracils are more likely than uracil to undergo a rare tautomeric change and then to resemble cytosine in pairing with a complementary base during nucleic acid replication (Freese, 1959). Mutation of DNA by 5-bromouracil (Litman and Pardee, 1956) may result from such a rare miscoding, and a similar ability to mutate RNA-coded RNA can be predicted for 5-FU from its apparent mutation of DNA-coded RNA, as deduced from the phenotypic reversion of certain rII phage T4 mutants (Champe and Benzer, 1962). In the DNA of such mutants, certain guanine-cytosine pairs have been replaced by adenine-thymine; the 5-FU is presumed to enter messenger RNA in place of uracil (being coded for by the mutant adenine), and there occasionally to behave like cytosine. On these occasions the original (wild-type) amino acid will be coded for, and the wild-type protein produced.

This paper reports that mutants form the majority of poliovirus progeny grown in appropriate concentrations of 5-FU. The mutations are detected as a permanently decreased ability to form plaques at supraoptimal temperatures. The efficiency of
plating at higher temperatures appears to be a more sensitive measure of 5-FU-induced miscoding than those used previously; some reasons for this are discussed.

MATERIALS AND METHODS

Virus was grown and assayed in embryo rabbit kidney (ERK) cells obtained by monolayer culture in medium CSV.6 (Cooper et al., 1959). The assays employed the agar cell suspension plaque method of Cooper (1961); the bicarbonate-free medium enabled various incubators to be used at different temperatures without special modification. Plates were enclosed in plastic boxes to minimize loss of moisture, and stained with the tetrazolium salt after 3 days at 36°-40°, or 6 days at 30°. The results of assays at different temperatures are expressed in terms of the efficiency of plating at a given temperature (e.g., eop-30°, eop-39.5°), and the efficiency of plating at, say, 30° equals the ratio of the titre obtained by incubation of assay plates at 30° to the titre obtained by incubation at 36°. In order to pick plaques for cloning virus strains, 10-cm petri dishes containing 10-50 3-day plaques were lightly sprayed with INT-glucose solution [15 mg of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride and 40 mg of glucose per milliliter of 80% ethanol] using a hand chromatogram spray. This method gives sharply contrasting plaques in less than half the time required by the aqueous stain (Cooper, 1959) without danger of mixing the clones.

Virus stocks. Various strains of poliovirus type 1 were used, whose growth, derivation, and temperature dependence are described by Cooper (1963). Many of these strains had been kindly donated by Dr. A. Lwoff, Institut Pasteur, Paris, France. Virus grown in presence of 5-FU is referred to as 5-FU virus.

5-Fluorouracil was obtained by the generosity of Roche Products Ltd. and was used without further treatment.

RESULTS

The Effect of Growth in 5-FU on the Plating Efficiencies of Poliovirus Strains

Table 1 shows a representative experiment in which 5-FU (4 mM) was present during the growth of four strains of poliovirus. These strains differed in their abil-

<table>
<thead>
<tr>
<th>Poliovirus strain</th>
<th>5-FU (mM)</th>
<th>40-Hr yield (PFU/ml × 10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Yield in 5-FU&lt;sup&gt;a&lt;/sup&gt;</th>
<th>eop&lt;sup&gt;b&lt;/sup&gt; of yield at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40°</td>
</tr>
<tr>
<td>VS.41</td>
<td>0</td>
<td>105</td>
<td>0.70</td>
<td>—</td>
</tr>
<tr>
<td>VS.41</td>
<td>4</td>
<td>10</td>
<td>0.21</td>
<td>—</td>
</tr>
<tr>
<td>BE</td>
<td>0</td>
<td>70</td>
<td>17 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>0.71</td>
</tr>
<tr>
<td>BE</td>
<td>4</td>
<td>2.1</td>
<td>&lt;5 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>0.071</td>
</tr>
<tr>
<td>VS</td>
<td>0</td>
<td>30</td>
<td>—</td>
<td>0.12</td>
</tr>
<tr>
<td>VS</td>
<td>0.15</td>
<td>0.50</td>
<td>—</td>
<td>0.02</td>
</tr>
<tr>
<td>VS.35</td>
<td>0</td>
<td>60</td>
<td>—</td>
<td>0.025</td>
</tr>
<tr>
<td>VS.35</td>
<td>4</td>
<td>0.12</td>
<td>0.20</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

<sup>a</sup> Monolayers, comprising 3 × 10<sup>4</sup> ERK cells and formed in Eagle's medium plus 5% calf serum, were inoculated at room temperature with sufficient virus in 0.5 ml PBS to infect 10-50% of the cells. After 30 minutes' adsorption the inoculum was removed and the monolayers were washed with 10 ml of PBS and incubated at 30° with 5 ml of Eagle's medium containing 0.1% bovine plasma albumen (and 5-FU where appropriate). The cultures were frozen after 40 hours, when cytopathic effect was complete in all control cultures; in cultures containing 5-FU, about 90% of the cells appeared morphologically unaffected. The 40-hour yield was determined by plaque assay at 36°.

<sup>b</sup> Percentage of yield obtained in the absence of 5-FU.

<sup>c</sup> Efficiency of plating, expressed as the ratio of plaque count obtained on incubation of assay plates at the indicated temperature to the plaque count obtained at 36°.
TABLE 2  
Effect of Nucleosides on the Changes Induced by 5-FU

<table>
<thead>
<tr>
<th>Additives</th>
<th>Control 5-FU (4 mM)</th>
<th>5-FU (4 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40-Hr yield PFU/ml X 10⁸</td>
<td>40-Hr yield PFU/ml X 10⁸</td>
</tr>
<tr>
<td></td>
<td>exp of yield at 39.5°</td>
<td>exp of yield at 39.5°</td>
</tr>
<tr>
<td>Nil</td>
<td>1.3 0.48 1.0 0.77 0.13</td>
<td>1.0 6.0 6.6 0.22</td>
</tr>
<tr>
<td>Uridine</td>
<td>0.9 0.48 6.0 6.6 0.22</td>
<td>1.06 0.52 8.3 7.8 0.17</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.76 0.54 14 18.5 0.30</td>
<td>0.76 0.54 14 18.5 0.30</td>
</tr>
<tr>
<td>Uridine plus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytidine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Monolayers of ERK cells were infected in darkness with VS.41 which had been grown in 4 μg/ml of neutral red (see text), incubated and harvested as described in Table 1. After the inoculum had been washed away, they were overlaid with Eagle's medium plus 0.1% bovine plasma albumen, containing the 5-FU and/or nucleoside as indicated. The 40-hour yield was determined by plaque assay at 36°.

To see whether the efficiencies of plating of 5-FU VS.41 (Table 1) might be spuriously high because of such masking by unecplased inoculum, two monolayer cultures were infected in darkness with a light-sensitive stock of VS.41 [grown in 4 μg of neutral red per milliliter (Wilson and Cooper, 1962)]. After 30 minutes' adsorption at 36° the cultures were irradiated with white light, washed and overlaid at 36° with 5 ml of Eagle's medium plus 0.1% bovine plasma albumen; one overlay contained 4 mM 5-FU. The results are shown in the first line of Table 2. In presence and absence of 5-FU, the 40-hour yields were, respectively, 2 and 225 PFU/cell and the eop-39.5° were 0.13 and 0.48. Thus the removal of unecplased inoculum virus does not appreciably alter the previous estimations of the 5-FU-induced change in plating efficiency of VS.41.

In other cultures in the same experiment (Table 2), uridine and cytidine (1 mM) produced some counteraction both of the inhibition of yield and of the decrease of eop-39.5° induced in VS.41 by 4 mM 5-FU. A mixture of both nucleosides was more effective than either alone. However, the apparent effectiveness of cytidine may only reflect its deamination to uridine, since this occurs readily in HeLa cells (B. McAuslan, personal communication).

The Permanent Nature of the Decrease in Plating Efficiency of 5-FU Virus

Two different stocks of VS.41 grown in 4 mM 5-FU were subcultured on different occasions by infecting washed monolayers with 0.2 ml of the stock. After 1 hour's adsorption at 20°, the inoculum was removed as thoroughly as possible, the cultures were washed with 20 ml of PBS, drained again, and then overlaid with medium CSV.6 at 36°. The cultures were frozen for assay when the cytopathic effect was most extensive (36–48 hours), and the resultant tissue culture fluid was used as the
inoculum to repeat this passage procedure. Altogether 9 such subcultures, all in the absence of 5-FU, were made of these two 5-FU stocks. The 9 subcultures included 2 series, one of 3 and the other of 4 serial subcultures; in the series of 4, the original 5-FU inoculum virus was diluted at least 10^{-10}. Two findings emerged. (1) The first and subsequent subcultures of 5-FU virus gave the same yields (in PFU/cell) as did parallel subcultures of normal VS.41. (2) The $eop$-39.5° of the progeny from all 9 subcultures was indistinguishable from that of the original 5-FU virus and was about 0.25 of the $eop$-39.5° of control VS.41 (subcultured and assayed in parallel) which had had no contact with 5-FU. Thus the $eop$-39.5° of VS.41 was permanently decreased by one subculture in 5-FU.

Single subcultures in the absence of 5-FU were made, using as inoculum about $10^3$ PFU from each of the BE and VS.35 stocks (grown in presence and absence of 5-FU) which are described in Table 1. After 5 minutes' adsorption at 20°, the inoculum fluid containing the 5-FU was washed away; the monolayers were overlaid with CSV.6, and the cultures were harvested after 40 hours at 36°, when the cytopathic effect was extensive. The progeny comprised a total of $10^8$ PFU in each case, equivalent to 10²-fold increases; the $eop$-38° of the BE progeny were, respectively, 0.63 and 0.29 for control and 5-FU virus, and the $eop$-38.5° of the VS.35 progeny were 0.09 and 0.03. Hence the changes induced by 5-FU are also permanent in strains which are already quite heat defective.

In another subculture series, 5-FU VS.41 ($eop$-39.5° equal to 0.23) was itself subcultured in presence of 4 mM 5-FU. As before, parallel subcultures in absence of 5-FU produced normal yields, and the $eop$-39.5° of the progeny was that of the inoculum 5-FU virus (0.2 to 0.3) rather than that of the parental VS.41 (0.65 to 0.80). In the presence of 5-FU, the cytopathic effect became less evident on successive subculture and yields were reduced to successively lower values, the third serial subculture in 4 mM 5-FU yielding less than $10^{-8}$ of the titres obtained in absence of 5-FU. However, the $eop$-39.5° of none of the subcultures differed from that of the 5-FU virus. Similarly, 3 alternate subcultures in presence and absence of 5-FU to maintain adequate titres also failed to alter the $eop$-39.5° from that of the inoculum 5-FU virus. It is presumed that in these cases an equilibrium was reached between the continued induction of heat-defective progeny and the selective inhibition by 5-FU of the growth of this progeny.

As discussed below, these results all indicate that 5-FU is highly mutagenic for poliovirus. In order to confirm that the 5-FU progeny do indeed contain a large proportion of heat-defective (hd) mutants, plaques were picked from a 5-FU-derived stock and from an untreated stock subcultured in parallel, and the properties of the plaque isolates were examined. The virus stocks were prepared as follows. A stock of VS.41 was grown at 40°, and assayed at 40°. A large well-isolated plaque was picked, and a clone was grown from it at 40°. The $eop$-40° of this clone was 1.0 to 1.3 (hd+); the clone was subcultured at 36° in monolayers in absence ("control") and in presence of 1 mM 5-FU, the cultures being frozen after 9 hours' growth. The control and 5-FU stocks were each subcultured once more for 1 cycle of growth at 36° in absence of 5-FU (the cultures being thoroughly washed after adsorption of inoculum) to make a final control stock and a "5-FU derived" stock. These last two stocks were assayed at 36°, and several hundred single plaques were picked from both into 1 ml PBS from 10-cm petri dishes containing 20-50 plaques (1-3 mm diameter). After standing overnight at 4°, a loopful of each plaque fluid was placed onto each of duplicate prepoured agar cell suspension plates, which were then incubated for 3 days, one duplicate at 36° and the other at 40°; 5 loopfuls were accommodated on one 5-cm petri dish. Heat-defective mutants were revealed as showing a "spot" of lysis or cluster of plaques at 36°, but none or very few at 40°.

The results were as follows. Of 264 plaque isolates tested from the 5-FU derived stock, 238 were hd+ and 26 were hd; this property was proved by the assay of stocks grown from each hd isolate, in which
their eop-39.5° varied from 0.7 to 10^-4. Their eop-39.5° was unchanged after recloning, and the eop-39.5° of the several hd+ isolates tested remained greater than 1.

The proportion of hd mutants in the progeny grown in 1 mM 5-FU was accordingly at least 10%, a value to be expected from the changes in eop-40° observed after growth in 1 mM and 4 mM 5-FU. Of 176 plaques tested from the final control (hd+) stock, 3 were heat defective, an isolation rate significantly less than that of the 5-FU virus. Nevertheless, an isolation rate of 3/176 suggests that the spontaneous rate of the hd type of mutation is high. As will be discussed below, the disturbance of growth processes by supraoptimal temperatures may be expected to be a sensitive test for “missense” mutations, in which one amino acid is replaced by another.

The Independence of the Genetic Factors Controlling the eop-30° and the eop-40°

It may be noted from Table 1 that the eop-30° of the three related strains VS.35, VS, and VS.41 decrease as the eop-39.5° or the eop-40° increase, whereas the eop-30° of the unrelated strain BE is much higher than would be expected from its eop-39.5°. Strains VS.35 and VS.41 had been adapted from VS to growth at 35° and 41°, respectively (Lwoff, personal communication), and the exception provided by BE suggests that the eop-30° and the eop-40° reflect two independent genetic factors (rather than two expressions of a single pleiotropic factor), which nevertheless are both altered by selection during adaptation to a new growth temperature. Such a coselection of independent characters during temperature adaptation has been shown in poliovirus for the characters of rt value and urea resistance (Cooper, 1963). The independence of eop-30° and eop-40° is also indicated by their different response to 5-FU (Table 1).

DISCUSSION

The main finding from these experiments is that one subculture of a strain of poliovirus in 4 mM 5-FU is sufficient to change permanently a certain heritable character of the strain. The ability to form plaques at supraoptimal temperatures is decreased, i.e., the strain becomes more defective in growth at high temperatures. This change cannot result from the selection of preexisting hd mutants, since it is shown that 5-FU inhibits the growth of hd strains more than that of strains which grow well at high temperatures. Opportunities for selection by thermal inactivation of hypothetical thermostable but heat-defective 5-FU progeny were generally not available. It is therefore concluded that the 5-FU directly causes mutation of the virus, presumably by the miscoding mechanism outlined in the Introduction. The relatively large drop in plating efficiency (two- to tenfold after growth in 4 mM 5-FU) indicates that the mutation rate must be very high: in at least some cases more than half of those progeny which would have formed plaques at the higher temperature have lost this ability. This is confirmed by the high isolation rate of hd mutants of at least 10% from progeny grown in 1 mM 5-FU.

The nature of the physiological expression of the 5-FU-induced mutations remains to be seen. However, since they have been revealed by defects in growth at high temperatures, it is probable that the mutations result in the synthesis of defective gene products whose defects are apparent only at higher temperatures. Certain temperature-sensitive mutants of Neurospora produce a tyrosinase which is more heat labile than that of the parental strain (Horowitz and Fling, 1953). Also, alkaline phosphatase synthesized by Escherichia coli in presence of 5-FU retains its activity but is more heat labile than the normal enzyme (Naono and Gros, 1960). There is evidence that mutations with the heat-defective type of expression are of very common spontaneous occurrence in nature (Langridge, 1962).

A decrease either in thermostability or in ability to polymerize with other proteins or in correctness of folding during synthesis of proteins must indeed be a very common result of the “missense” mutations to be expected both of 5-FU and of many spontaneous genetic changes. Since the configuration of proteins depends upon critically placed hydrophobic bonds, together with
salt and disulfide linkages, between various of the amino acids (Kauzmann, 1959), a replacement of any amino acid by another will be unlikely to result in the same type of cross-linkage and usually will accordingly weaken the nonprimary structures. The structures will then become more dependent on the hydrogen bonding that exists between peptide links, and hence become more easily changed by a small rise in temperature. Only a very small change in nonprimary structure, perhaps originating at points distant from the “active site” (Monod et al., 1963), may suffice to alter the molecules’ metabolic efficiency.

This implies that the target for the $hd$ type of expression of missense mutations may be comprised by substantial parts of the whole protein molecule, in contrast to other types of expression in which, for example, only the results of amino acid replacements in the active site itself may be apparent. Such a difference in effective target size may explain the finding, described above, that concentrations of 5-FU that affect the $eop-40^o$ do not detectably affect the $eop-30^o$. Like the $eop-40^o$, the $eop-30^o$ is in our hands a relatively stable character which is reproducible in a given strain, but which can be varied by adaptation of that strain to new growth temperatures. It may also explain in part the common occurrence of $hd$ mutants.

The likelihood that naturally occurring heat-defective strains of poliovirus make heat-defective gene products of the type outlined above is well supported by the effects of temperature, D$_2$O, and urea solutions on the growth of these strains (Lwoff and Lwoff, 1961, 1962), and some explanations of these effects have already been suggested (Lwoff, 1962). The finding reported in the present paper of 3 $hd$ mutants among 176 plaques picked from a normal $hd^+$ clone indicates that the spontaneous rate of missense mutation in RNA is probably quite high.

The inhibition of poliovirus growth at $36^o$ by 5-FU may well be explained in part by the induction of mutants that are defective even at that temperature, and that therefore behave as lethal mutants. It is also likely that gene products (conceivably coded for either by the virus or by the host cell) are rendered phenotypically heat defective by the incorporation of 5-FU into cellular transfer or messenger RNA. These concepts are supported by the observation that the degree of poliovirus growth inhibition by 5-FU depends on the degree of defectiveness of the strain: the effects of two sources of heat-defective gene products (i.e., an intrinsically defective strain plus 5-FU incorporated into cellular and/or viral RNA) might well be expected to be additive.

The apparent lack of effect of 1 mM 5-FU on the composition of viral coat proteins (Munyon and Salzman, 1962; Holoubek, 1963) may reflect one or more of the following possibilities: (a) the coat protein is likely to be coded for by a relatively small and hence less vulnerable portion of the genome; (b) the structural units for the coat of the 5-FU virus may be selected during maturation preferentially from those molecules that are most nearly normal; (c) the 5-FU progeny is likely to contain many different mutants in which different amino acids are affected: the aggregate effect of many individual replacements of one amino acid by another is not likely to be detectable in an uncultivated population.

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I am grateful to Roche Products Ltd. for a generous gift of 5-fluorouracil, and to Miss P. Herde for very competent technical assistance.

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Cooper, P. D., Wilson, J. N., and Burt, A. M.


Synchrony and the Elimination of Chance Delays in the Growth of Poliovirus

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SUMMARY

The effect of multiplicity of infection on the growth kinetics of poliovirus suggests that the eclipse period usual for singly infected cells (3.6-3.8 hr) includes variable delays averaging about 1 hr. These delays are overcome at multiplicities above 3, and are sometimes spontaneously absent. They are ascribed largely to chance effects, and lead to markedly asynchronous maturation of virus; in their absence, maturation is almost synchronous.

The kinetics of acid-irreversible eclipse and of the development of antiserum resistance show that about half of the delays must occur during viral penetration; in support of this, virus growth initiated with infective RNA is 0.5 hr less delayed than that of intact virus, although otherwise similar. However, infective RNA synthesis was not detected earlier than 2 hr after infection, even in the absence of chance delays.

INTRODUCTION

The events to be observed during a single cycle of virus growth usually form a reproducible sequence. However, even if the cells are infected simultaneously, all cells may not reach a given part of the sequence at the same time (Cairns, 1957). Such asynchrony occurs in many viral systems, and may at times hinder the study of virus growth as much as non-simultaneous infection of the cells. One cause of asynchrony is the presence of chance hesitations between growth steps ('random or variable delays') and it is important in studying virus growth to know the average duration of the viral processes as compared with such delays. It is theoretically possible, for example, that the eclipse period results solely from very large random delays.

This work investigates the extent of delays and asynchrony in the growth of poliovirus in ERK cells, and ways of avoiding them. Of the eclipse period of 3.6 hr which is usual at single multiplicity of infection, nearly 1 hr may be regarded as occupied by random delays. These delays result in substantial asynchrony of virus maturation, and a considerable portion of them appears to occur during stages of penetration. However, random delays and asynchrony are sometimes absent, probably because of factors affecting the state of the cells, which are not understood. Random delays are also eliminated by large multiplicities of infection, which result in considerable synchrony of viral maturation and a reproducibly short eclipse period of 2.6-2.7 hr.

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METHODS

**Virus.** Poliovirus type 1 (strain Brunenders) was used; virus and infective cells were assayed by the agar cell-suspension plaque method (Cooper, 1961). Infective RNA assays used the procedure described by Cooper (1962). All host cells were from the ERK line grown in medium CSV6 (Cooper, Wilson & Burt, 1959).

**One-step growth method.** ERK cells were resuspended from bottle cultures with trypsin (2.5 mg./ml.) plus EDTA (2 mg./ml.) to give a monodisperse suspension, and maintained overnight as a suspension culture at 36°. Cells were infected to various multiplicities in suspension at 0°, washed and added to medium at 37°. The pH was controlled at pH 7.3 by replacement of glucose with galactose and by gassing with 5% CO2 in air. The cells were kept in suspension by a 'New Brunswick' type of rotary shaker. The final cell suspension was assayed for total cells (5–10 x 10^5/ml.), infective cells (50–80% of total cells when fully infected) and virus free (less than 5% of infective cells); < 10% of the cells were in clumps of 2 or more, and < 5% were non-viable. The multiplicity of infection was determined from assays of the virus added and of the virus recovered after washing the cells. Samples were taken at intervals during virus growth into 2 mg./ml. deoxycholate for assay of cell-associated mature virus. Growth is expressed in terms of plaque-forming units (p.f.u.) produced per infective cell, and the eclipse period equals the time from addition to warm medium to the time of maturation of 1 p.f.u. per infective cell.

**Sampling procedure for determination of yield per yielding cell.** Portions of infected cell suspensions were taken during 1-step growth, chilled and diluted to 5, 10 and 20 infective cells/ml.; 0.05 ml. samples of these dilutions were added to each cup of an 80-cup haemagglutinin tray, and then frozen in solid CO2 and thawed. Fresh cells were added to each cup (0.05 ml. containing 10^6 cells), followed by 0.1 ml. of molten agar medium. The cup contents were very rapidly mixed, removed and dropped on to chilled agar base layers contained in 4 in. Petri dishes; the drops were spread somewhat before they set. Eight samples were accommodated per dish, and 600–700 samples were plated in 4–5 hr on 80 Petri dishes. The plates were then incubated as for a normal plaque assay, when the plaques developing in each sample could be counted; only those dilutions in which about 50% of the samples contained plaques were selected for counting.

RESULTS

**The effect of multiplicity of infection on the growth of poliovirus.** If there are variable delays in a viral growth process caused purely by probability effects, then increasing the multiplicity of infection should decrease the overall chance of delay for any given infected cell (Cairns, 1957). The eclipse period at single multiplicity was delayed to 3.6 hr but increasing the number of adsorbed particles decreased the eclipse period to 2.6–2.7 hr (Fig. 1). These values were quite reproducible, and eclipse periods were never less than 2.6 hr however high the multiplicity (maximum tested equals 80). Figure 2 suggests that the eclipse periods are made up of a component of up to 1 hr which is variable with multiplicity plus an variable component of 2.7 hr. The shortening of the eclipse period was negligible above a multiplicity of 3. An arithmetical plot (Fig. 3) of the data of Fig. 1 shows that the rate of maturation tends to be constant and that, at very low multiplicities, virus matures
Synchrony in the growth of poliovirus

more slowly. Similar curves were found for influenza virus under conditions of delay and asynchrony (Cairns, 1957). Darnell (1958) and Howes (1959, a, b) reported that higher multiplicities increased the growth rate of poliovirus.

Fig. 1. One-step 'delayed state' maturation curves of poliovirus in suspended ERK cells at 37°. Six cultures from one batch of cells were simultaneously infected with various multiplicities of virus, which are indicated on the figure. The short vertical lines join each curve at the point where the internally matured virus equals the infective cell count (end of eclipse period).

However, in rather less than half of our experiments the growth rate at single multiplicity of infection did not show any delay (Fig. 4): the eclipse period was 2-7 hr, maturation was rapid and exponential in rate, and increasing the multiplicity had no effect. The reasons for this lack of delay are not known, but are suspected to lie in the metabolic state of the cell. In the growth curves of Fig. 4, the increase of infective RNA was also measured for the two cultures, and was the same for both. The RNA curve from the lower multiplicity culture is shown, and indicates
that, even in the absence of delay, an increase in infective RNA was not detected earlier than 2 hr after infection. This aspect is dealt with more fully in the accompanying paper (Cooper, 1964).

Fig. 2. The relation for poliovirus between the reciprocal of the multiplicity of infection (1/M) and the eclipse period. One experiment (●) is that of Fig. 1; the other (○) is a replicate. Multiplicity of infection in this case equals the average number of particles adsorbed per infected cell (minimum equals 1), and is calculated assuming a Poisson distribution of virus among cells.

Fig. 3. One-step 'delayed state' maturation curves of poliovirus in suspended ERK cells at 37°. The data are the same as those of Fig. 1, and the multiplicities of infection are indicated on the figure.

Synchrony and asynchrony in maturation of poliovirus. The synchrony of maturation was examined by the following direct method, using delayed cultures and cultures in which delay was spontaneously absent or was eliminated by a high multiplicity of infection. One-step growth experiments were performed in which samples of intact cells were taken at times covering the estimated end of eclipse (2.5–4 hr). A series of dilutions of each of these samples was made, and 0.05 ml. of each dilution was added to every cup of an 80-cup haemagglutinin tray. The dilutions were such that, for each sample, at least 1 tray contained an average of about 1 infective cell per two cups. After freezing the samples in the trays and thawing to liberate the virus, the contents of each cup were plated by the procedure already described. The assays from those trays in which about half the cups yielded no or negligible virus enabled the infective virus content of individual cells to be calculated. Three representative experiments are shown in Table 1. In Exp. 1 the eclipse period at single multiplicity of infection was 2.6 hr and virus maturation was rapid and almost exponential, and so random delays were presumed to be absent. The degree of asynchrony was in fact very small; the distribution of virus
among cups was approximately Poissonian, and the number of cups yielding virus at the end of the eclipse period was almost that to be expected from the infective cell assay, so that almost all potentially yielding cells contained some virus at this time.

Table 1. The distribution of poliovirus among cells of three cultures experiencing different random delay effects

The one-step growth and sampling procedures are described in the Methods.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>1</th>
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<th>3</th>
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<tr>
<td>Multiplicity of infection</td>
<td>0-01</td>
<td>0-01</td>
<td>5</td>
</tr>
<tr>
<td>Eclipse period (hr)</td>
<td>2-6</td>
<td>3-7</td>
<td>2-6*</td>
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<tr>
<td>Presumed state</td>
<td>Non-delayed</td>
<td>Delayed</td>
<td>Delay overcome by high multiplicity</td>
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<table>
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<th>Time of sampling (hr)</th>
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<th>3-0</th>
<th>5-0</th>
<th>4-0</th>
<th>2-0</th>
</tr>
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<table>
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<tr>
<th>Yield per yielding cell</th>
<th>(a) From average of positive samples</th>
<th>1-25</th>
<th>1-4</th>
<th>100-200</th>
<th>3-0</th>
<th>6-0</th>
</tr>
</thead>
</table>

| (b) Expected from growth curve | 1-1 | 3-3 | 160 | 2-5 | 6-5 |

<table>
<thead>
<tr>
<th>Plaques per sample (each sample is presumed to contain only 1 infective cell)</th>
<th>p.f.u.</th>
<th>No. of samples</th>
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<tr>
<td>0</td>
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<tr>
<td>1-3</td>
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<td>4-10</td>
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<td>0</td>
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<tr>
<td>&gt; 100</td>
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</table>

* A replicate experiment performed simultaneously but at single multiplicity had an eclipse period of 3-6 hr.
† Released virus.

In Exp. 2, the maturation rate was slow and the eclipse period was 3-7 hr; random delays were presumed to be present. This experiment showed much more asynchrony than the first in that the yields per cell varied widely and the proportion of cells yielding no virus was higher than that to be expected from the average yield per cup. In Exp. 3, the culture examined had a multiplicity of 5, and an eclipse period of 2-6 hr, while a replicate culture at single multiplicity grown simultaneously had an eclipse period of 3-6 hr. Hence it is presumed that these cultures were in a state in which each adsorbing particle would be subject to random delays but that such delays were overcome for the infective cell by a high multiplicity. Table 1 shows that overcoming the delays in this way also removed the asynchrony. Thus delayed cultures were highly asynchronous in maturing virus, while cultures with little intrinsic delay or in which delay was overcome by high multiplicity were much less asynchronous.

Delays in entering acid-irreversible eclipse. Of several stages detected during the penetration of poliovirus, the transition to non-recoverability of infectivity with pH 2-5 buffer ('acid-irreversible eclipse') appears to be the last (Fenwick & Cooper, 1962). The rate of acid-irreversible eclipse in the system used (Fig. 5) shows that the time taken to inactivate the virus in this way can occupy a significant portion of the eclipse period, as 50% of the particles in Exp. A were delayed by at least 0-5 hr. However, the rate of eclipse was not reproducible between experiments (in Fig. 5, curve B, the 50% time was only 0-15 hr) indicating that delays involved in eclipse were variable and were less in some experiments than in others.
Delays in development of antiserum resistance. To see whether delays were introduced at a stage earlier than acid-irreversible eclipse, the rates of development of antiserum resistance of the infective cell were compared at different multiplicities of infection. Multiplicity had a marked effect and the development of antiserum resistance was faster at a high multiplicity (Fig. 6). With 10 p.f.u./cell, the transition of almost all cells occurred within a few minutes. As the maximum hastening of antiserum resistance by high multiplicity was only 20–30 min., this source of delay possibly accounts for rather less than half of the delays usually present. However, in several single-multiplicity growth experiments all infective cells became resistant to antiserum in 1–5 min., indicating that the delays involved in this growth step are also variable between, as well as within, experiments.

Comparison of growth curves with virus and with infective RNA as inocula. If the uncoating of the virion leads to variable delays in the growth cycle, then infection of the cell with uncoated genetic material (infective RNA) should reduce this delay. The eclipse period and the time taken to release 1 p.f.u./cell were both 0.5–0.8 hr shorter when using infective RNA than when using intact virus at single multiplicity (Fig. 7). Hence a considerable portion of the delay occurs during the uncoating of the virus.

A control experiment was necessary for the interpretation of Fig. 7. This was because independent experiments with infective RNA labelled with $^{32}$P showed that as much as 5% of the RNA was taken up by the cells when the RNA was added at
Synchrony in the growth of poliovirus

high concentration. Thus it was possible that the virus deriving from infective RNA in Fig. 7 did not contain newly synthesized RNA, but inoculum RNA which had been 'recoated'. This possibility was disproved by preparing two replicate batches of virus labelled with $^{32}$P, using non-radioactive infective RNA (extracted from virus at a concentration of $10^{10}$ (p.f.u./ml.) and non-radioactive intact virus respectively as inocula. The procedure for virus growth and purification described by Fenwick & Cooper (1962) was used; the viral radioactivity was isolated from both preparations by means of a potassium tartrate density gradient, and their specific activities were found to be almost identical.

Fig. 6. Effect of multiplicity of infection of poliovirus on the development of antiserum resistance of suspended ERK cells at 37°. Multiplicities were 10 (○), 2-5 (△), and 0-01 (●). Infected cells were diluted at intervals into sufficient antiserum to neutralize 99% of free virus in 10 min. at 4°. After 90 min., the cells were diluted free of antiserum and plated as for virus. The cells surviving as infective centres are expressed as a percentage of cell samples containing no antiserum.

Fig. 7. One-step growth curves of poliovirus initiated by infective RNA (○ = cell-associated virus, or CAV; △ = released virus) and by intact virus (● = CAV; ▲ = released virus). Both sets of cultures had been treated identically before infection, including washing with 0-8 M-Na$_2$SO$_4$ at pH 8-0; they were infected as monolayers at 15°, washed rapidly several times with PBS at 37° in a room at 37°, resuspended with trypsin and versene mixture, and washed before adding to warm growth medium. Time zero equals the time of transition to 37°; care was taken that this transition was sharp and permanent.

DISCUSSION

These data indicate that the normal single multiplicity eclipse period of poliovirus, 3-6–8-8 hr in the system studied, includes variable delays of about 1 hr. At least half but not all of these delays occur during penetration of the virus, probably after the reversible 'neutralization' of virus by cells which occurs with little delay (Fenwick & Cooper, 1962). The term 'penetration' is used here to include all stages up to the final uncoating of the genetic material. The delays lead to considerable asynchrony of maturation. Howes (1959a, b) estimated that there was a total spread of
2–3 hr for the termination times of individual eclipse periods of poliovirus in his system. However, delays and concomitant asynchrony are sometimes spontaneously absent, or can be overcome by a high multiplicity of infection; criteria of such effects are a short eclipse period and rapid maturation. Variations in the extent of delay may explain the differences in kinetics of poliovirus growth apparent between other reports (Dulbecco & Vogt, 1955; Darnell, 1958; Howes, 1959a, b; Holland, Hoyer, McLaren & Syverton, 1960; Darnell, Levintow, Thorén & Hooper, 1961). It is clearly desirable, particularly in biochemical studies of virus growth, to eliminate asynchrony during the infective process, and this now appears to be feasible by use of the method described above. An implication of these findings is that practically all of the 2–7 hr minimum eclipse period of poliovirus must be occupied by viral growth processes, rather than by chance hesitations between processes.

It should be noted that the eclipse periods would also be shortened by high multiplicities if each adsorbing particle were able to replicate autonomously but without delays (Cooper, 1958). In this case the variety of individual multiplicities of infection in a culture would yield considerable asynchrony, again resembling the effects of random delay. However, the acid-irreversible eclipse curves and growth curves resulting from infective RNA indicate that much delay must be present during penetration, and hence that the contribution of autonomous growth to the effects found with poliovirus must be correspondingly limited. The fact that increasing the multiplicity above 3 does not further shorten the eclipse period also indicates that fully autonomous growth is not permitted.

The excellent assistance of Mr H. Cumming and Miss J. Constable is gratefully acknowledged.

REFERENCES
The Kinetics of the Appearance of Poliovirus Ribonucleic Acid

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SUMMARY

One-step growth conditions were used in which cells began replication of poliovirus RNA almost synchronously, and in which factors limiting the rate of replication appeared to be absent. RNA of uneclipsed virus was destroyed by using light-sensitive inocula. Under these conditions, ribonuclease-sensitive infective RNA was detectable in unchanged amount for the first 2 hr of infection, at which time replication began abruptly; the increase of poliovirus RNA was geometrical for a further 1–2 hr.

INTRODUCTION

Where the kinetics can be determined, the rates of increase of several viral nucleic acids (measured chemically or by infectivity) appear in one-step growth experiments to be more or less constant with time. However, in phage T₂ (Hershey, Dixon & Chase, 1953) a genetic experiment (Luria, 1951) showed that DNA replication depends on a geometric mechanism. Rate-limiting factors may, therefore, have transformed a presumptively exponential rate of increase into an apparently linear one. Another complication having the opposite effect is that individual cells may not begin nucleic acid replication synchronously. In this case, an exponential rate of increase in the proportion of cells which begin replication could transform a presumptively linear rate of increase of nucleic acid into one which was apparently exponential in the initial phase.

This paper reports experiments on the rate of increase of poliovirus RNA under conditions lacking these complications. Random delays were largely absent so that maturation began almost synchronously (Cooper, 1964). Cells were infected with a light-sensitive inoculum (Wilson & Cooper, 1962). Illumination after a short 'pulse' of penetration gave additional synchrony and destroyed the RNA of virus uneclipsed during this period (Wilson & Cooper, to be published): the very early kinetics could be examined without their being obscured by excess of non-productive RNA. The appearance of new infective RNA began sharply at 2 hr; the increase was exponential for the next 2 hr, so that rate limiting factors were also absent.

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Virus. Poliovirus type 1 (strain Brunenders) was used, grown and assayed in ERK cells (Cooper, Wilson & Burt, 1959; Cooper, 1961). Infective RNA was extracted and assayed by the method described by Cooper (1962); all RNA assays used two or three 3-fold dilutions, 4 in. Petri dishes and four replicates per dilution.

One-step growth method. The method used was described by Cooper (1964); mono-disperse cells were infected to an adsorbed multiplicity of 10 at 0°, washed and added to medium at 37° at zero time. Serum was omitted from the medium. The cells were maintained in suspension by a rotary shaker. Inoculum virus was grown in neutral red, 4 μg./ml. and all operations including addition to warm medium were performed under a red light (Wilson & Cooper, 1962, 1963); after 5 min. at 37° the cultures were strongly illuminated for 10 min. with white light (proportion of virus surviving under these conditions equals 10^-3). Samples were taken for infective cell and free virus assay, and at intervals into 2 mg. deoxycholate/ml. for cell associated mature virus assay (no free virus was present, and no cell associated virus was found up to 2-3 hr after infection), and into aqueous phenol for extraction of RNA.

For all cultures the eclipse periods (time after infection of appearance of 1 intracellular p.f.u. per infective cell) were 2.6-2.7 hr and maturation was rapid and exponential. These criteria were regarded as essential to indicate the absence of random delays and asynchrony (Cooper, 1964).

Fig. 1. One-step growth curve of poliovirus infective RNA, plotted semilogarithmically (○, left-hand ordinate) and arithmetically (●, right-hand ordinate). Uneclipsed inoculum virus was destroyed by use of light sensitive inocula, and virus maturation was approximately synchronous.

Fig. 2. Two replicate one-step growth curves of poliovirus infective RNA, expressed as a percentage of the 4-hr yield. Conditions as for Fig. 1.
Poliovirus RNA synthesis

RESULTS

An intensive examination was made of the rate of increase of poliovirus RNA between 1 and 3 hr after infection (Fig. 1). The eclipse period, as determined by assays of mature virus and infective centres (not shown), was 2-6 hr. This indicates that maturation was approximately synchronous (Cooper, 1964), and hence that all cells must have begun replication of viral RNA well before this time. More than 60% of the RNA found up to 2 hr was sensitive to ribonuclease when the cells were disrupted and treated with the enzyme before phenolic extraction. Infection with virus grown in 4 μg. neutral red/ml. followed by white light has destroyed 99.9% of the RNA of uneclipsed virus (Wilson & Cooper, to be published). Therefore this initial RNA represents input nucleic acid which was uncoated but not degraded or eclipsed. RNA synthesis began sharply 2 hr after infection and increased exponentially up to 4-0 hr (Fig. 2). Increases in infective RNA generally continued up to 5 hr in this system; the increase in infective RNA was $10^3$ to $10^4$ times the initial (2 hr) value, whereas the yield of virus rarely exceeded 800 p.f.u./cell. It is presumed that this excess of infective RNA reflects the RNA built into particles which do not register as plaque-forming units, together with any RNA which is not matured.

DISCUSSION

These data indicate that poliovirus RNA can be shown to increase geometrically, provided that adequate technical safeguards are taken. This may reflect a truly geometric mechanism, in which each progeny RNA molecule is able to act as a template for new molecules. This conclusion is supported by the observations that the proportion of poliovirus recombinants increases during the growth cycle (Ledinko, 1963), and that more than one molecule per cell of the RNA of the closely similar EMC virus can exist in double-stranded form (Montagnier & Sanders, 1963). The underlying mechanism, however, may be no more than pseudogeometric. For example, each progeny RNA molecule may induce the synthesis of some substance which increases the rate of replication, conceivably viral RNA polymerase.

I am grateful to Mr H. Cumming and Miss J. Constable for skilful technical assistance.

REFERENCES


Rescue of One Phenotype in Mixed Infections with Heat-Defective Mutants of Type 1 Poliovirus

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Accepted November 20, 1964

When cells are mixedly infected with two heat-defective (hd) poliovirus mutants at temperatures above those that are optimal for growth of these mutants, the virus yield is 4-14 times the sum of the yields of each mutant when grown separately under identical conditions ("leak" yields). The mixed infection yields are only one thousandth of the yield of wild type (hd+) under these conditions. The leak and mixed infection yields are 90-99% mutant rather than hd+, but only one of the mutants was detected in the mixed infection yields. Any rescue of the hd mutants by hd+ was too small to be detected.

INTRODUCTION

Conditional lethal mutations of bacteriophage T4, which prevent its growth either in certain hosts or at higher temperatures, have been used with great effect (Epstein et al., 1963) to map the T4 genome and to indicate many of its gene functions. The discovery that 5-fluorouracil produces many heat defective (hd) mutants in poliovirus (Cooper, 1964) suggests the possibility of a similar approach to the genetic analysis of a small animal ribovirus.

The hd mutants that have been isolated are now being examined in several ways. One way is to classify them by means of a complementation test, with the object of defining those groups of mutants which have defects in the same gene. This test showed that mixed infection at high temperature with many but not all pairs of hd mutants gave an enhanced yield. The enhancement was always very small, however.

This paper describes the complementation test and reports the results of some experiments in which the interaction between two of the mutants is examined in some detail with the object of defining optimal conditions. It is shown that, for this particular pair at least, the enhancement was not reciprocal within the sensitivity of the test.

METHODS

Temperature control. Well-circulated water baths controlled by Braun (Melsungen, Germany) Thermomix II mercury contact units maintained temperature to ±0.05°C; the most convenient monitors were clinical thermometers graduated in 0.1°C. Tubes were stoppered and were immersed to a depth of 3 cm in open racks; maximum temperature was reached within 3 minutes. Plaque assays of virus at high temperatures were made with a bicarbonate medium (Cooper, 1961) contained in screw-capped bottles immersed inverted in water baths in open baskets, in such a way as not to impede water circulation. In some experiments (indicated in the text) petri plates were enclosed in a sealed box that was immersed in a water bath at a supraoptimal temperature, but this method was discontinued because poor initial temperature control produced a spuriously high plaque count with the hd mutants.

Infectivity assays. The agar cell-suspension plaque method (Cooper, 1961) was used, with the human amnion strain U cells.
(Pohjanpelto, 1961) grown in medium CSV.6 (Cooper et al., 1959).

**Virus strains.** All virus strains were poliovirus type 1. Strains *hd-5* and *hd-19* are two of 26 heat-defective and doubly plaque-purified clones isolated from a stock of *hd* that was grown in the presence of 1 mM 5-fluorouracil (Cooper, 1964); *hd* is a clone grown at 40° from a large plaque picked from a 40° assay of strain VS.41, which itself is the Sabin vaccine strain LS adapted by Dr. A. Lwoff to grow at 41°. Both the leak and reversion rates are low but not zero in both *hd-5* and *hd-19.*

**Virus stocks.** The mutant and *hd* stocks used in these experiments were grown in monolayers infected with a multiplicity of 2-3 PFU/cell. After infection the cells were washed, overlaid with Eagle's medium containing 10% calf serum and 4 µg neutral red per milliliter, and incubated at 36° for 16 hours in darkness. All manipulations up to irradiation (see below) were done under a red safe light (Wilson and Cooper, 1963).

**Standard complementation test.** Barely confluent monolayers of 3 × 10^5 U cells were grown on the base of stoppered flat-bottomed tubes (20 mm diameter) by overnight incubation in 1 ml medium CSV.6; the tubes were always used standing upright. These cultures provided reproducible replicates. After immersion in ice water and removal of the medium, each tube received 3 drops of virus dilutions from pasteur pipettes calibrated to deliver drops of 0.02 ml. The tubes were shaken and stood for 90 minutes at 0°; the inocula were then removed, and the tubes were washed once with 2 ml of PBS. One milliliter of cold Eagle's medium containing 10% calf serum and 0.03% bicarbonate was added per tube, stockers were replaced, and the tubes were plunged into a water bath at the appropriate temperature. It is estimated that the cells reached water bath temperature within 1 minute. After 2-3 hours the cultures were irradiated with white light without removing them from the water bath, a treatment which reduced noneclipsed infectivity to <10^5 PFU per culture so as not to obscure the low yields obtained. After incubation for a further 4-5 hours the tubes were frozen for assay of infectivity. Tubes were usually in duplicate, and each experiment included controls of cultures infected with each mutant alone plus medium, to give the same inoculum dilution as in the mixed infections.

**Measurement of thymidine uptake by infected cells.** Replicate tubes containing 3 X 10^5 cells were completely infected with each virus stock in duplicate at 0°. The stocks used were clones isolated from a complementation experiment, and stocks of *hd-5, hd-19,* and *hd*; some tubes were left uninfected to serve as controls. The cells were washed, overlaid with 1 ml of medium and duplicate tubes were incubated at 37.0° and at 39.5°. After 3.75 hours' incubation, 2 equal drops of a solution of thymidine-2-C^14 (New England Nuclear Corporation, 0.1 mc/0.96 mg) were added per tube to give about 10^6 cpm per milliliter of culture; incubation was continued for a further 1.75 hours, when the cultures were abruptly chilled, the medium was drained off, and the cells were washed thoroughly 3 times with 2 ml PBS. Four ml of cold 10% trichloroacetic acid were then added to each tube, and after standing overnight at 4° the tubes were washed again with trichloroacetic acid. The tubes were then heated (20 minutes at 100°) and dried; the contents, dissolved at 100° in 0.2 ml 0.2 N NaOH, were transferred to planchettes and counted in an end-window counter. Duplicate cultures all agreed within a few counts per minute.

**Terminology used.** The leak refers to the ability of a mutant stock to produce some mutant progeny under the restrictive growth conditions, as distinct from reversion, which refers to the ability of purified mutant stocks to produce progeny that are wild type in respect of the character examined.

**RESULTS**

**The Thermal Growth Requirements of hd, hd-5 and hd-19**

Figure 1 compares the effect of temperature on the abilities of *hd*, *hd-5*, and *hd-19* to form plaques, and also shows the effect of temperature on the 6-hour yield of *hd* in liquid medium. It is seen that plaque production by *hd* decreases above 39.0°.
COMPLEMENTATION BETWEEN POLIOVIRUS MUTANTS

Fig. 1. Thermal growth requirements of the poliovirus strains described in this paper. Three curves (O, A, A) show the effect of temperature on plaque formation, plaque assay bottles of hd+, hd-5 and hd-19, respectively, being immersed for 3 days in water baths at the indicated temperatures. The fourth curve (●) shows the 6-hour yield of hd+ when grown in tube cultures at the indicated temperatures.

and is only 25% of the maximum value at 40.0°. The 6-hour yield of hd+ drops more rapidly and is only 4% of the maximum value at 40.0°. The mutations that gave rise to hd-5 and to hd-19 have had a very large effect on growth at high temperatures: at 38.9°, the plaque formation by both mutant strains is 2 to 3 × 10⁻³ of the maximum value, while at 39.0° that of hd-5 is 10⁻¹, and that of hd-19 is 2 to 3 × 10⁻⁴. The opposite differences in sensitivity of hd-5 and hd-19 above and below 38.9° are reproducible. The plaques produced by both mutants above 39° are small and indistinct; they are clearly distinguishable from those of hd+, which are large and sharp.

Mixed Infection with hd-5 and hd-19 at High Temperatures—Effect of Multiplicity of Infection

Table 1 shows the marked enhancement in yield that occurs when cells are mixedly infected with hd-5 and hd-19 and are then incubated at a temperature above the optimum for growth of these mutants. The replicate assays at 39.2° (in parentheses) show that the enhanced yields are predominantly mutant, not wild type. The yields from each mutant alone are also mainly mutant, and hence represent leak rather than content of wild-type revertant. As is to be expected, enhancement of yield is small where not all cells are infected. However, a phenomenon found in most experiments with many hd mutants is shown by the undiluted hd-19 stock; the yield is often less enhanced at higher multiplicities. The highest mixed infection yield was about 10⁻⁴ of wild-type yields, and 4-6 times the sum of the individual leak yields.

Table 1 also shows another consistent finding: the increase in multiplicity of infection of the mutant controls above that needed to infect all cells does not increase the leak yield. For this reason, and also because only 1 mutant may predominate in the mixed infection yield (see below), the true control value for comparing with mixed infection yields is probably less than the sum of the individual leak yields. However, since this “true value” cannot be arrived at, the sum will be used.

The Proportion of Each Mutant in the Mixed Infection Yield

In order to see whether the apparent complementation was fully reciprocal, i.e., whether both mutants appeared equally in the yield, a standard complementation test (duplicate tubes, 6 hours at 38.8°) was performed with approximately equal multiplicities of hd-5 and hd-19. The average mixed infection yield was 5.3 × 10⁴ PFU per 3 × 10⁵ cells, while the single infection yields were 1.1 × 10⁴ (hd-19) and 5 × 10³ (hd-5). Four-inch petri dishes were employed for the assays, and about 50 plaques per dish were present in the assay of the mixed infection yields; after spraying with stain (Cooper, 1964) plaques were picked at random and clones were grown from them.

Wild-type poliovirus inhibits the uptake of thymidine by cells. Strain hd-5 is defective in this inhibition at 39.5° but not at 37.0°, while hd-19 is not defective in this function.
TABLE 1

**Effect of Multiplicity of Infection on Mixed Growth of Poliovirus Strains**

\(hd-5\) and \(hd-19\) at 39.2°C

<table>
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<th>(hd-19) inoculum (PFU/cell)</th>
<th>(hd-5) inoculum (PFU/cell)</th>
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<tr>
<td>20</td>
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<tr>
<td>6</td>
<td>136 (5)</td>
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<td>89 (7)</td>
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</tbody>
</table>

| \(hd+\) (20 PFU/cell) | 144 (150) |

\* The conditions were those of the standard complementation test (see Methods), in tube cultures at 39.2°C. Tubes were irradiated with white light at 2.5 hours, and frozen for infectivity assay at 6.5 hours.

\* The figures in the table are the plaque counts on plates containing 0.1 ml of 10⁻¹ dilution of the 6.5 hour yields and incubated at 37°C; the figures in parentheses are plaque counts on replicate petri plates incubated in boxes immersed in a water bath at 39.2°C.

\* Plaques from 0.1 ml of 10⁻⁴ dilutions.

TABLE 2

**Effect of Infection with Clones Isolated from a Complementation Test on Cellular Uptake of Thymidine**

<table>
<thead>
<tr>
<th>Controls</th>
<th>Thymidine ³ uptake at 37.0°C</th>
<th>Thymidine ³ uptake at 39.5°C</th>
<th>Thymidine ³ uptake at 37.0°C</th>
<th>Thymidine ³ uptake at 39.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>48</td>
<td>57</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Uninfected</td>
<td>47</td>
<td>59</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>(hd-5)</td>
<td>11</td>
<td>3</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>(hd-5)</td>
<td>13</td>
<td>-2</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>(hd-19)</td>
<td>11</td>
<td>20</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>(hd-19)</td>
<td>8</td>
<td>23</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>(hd+)</td>
<td>15</td>
<td>26</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>(hd+)</td>
<td>10</td>
<td>24</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>

\* Procedure described in Methods.

\* Thymidine C¹⁴ cpm/3 X 10⁴ cells taken up in 1.75 hours; uninfected values represent observed uptakes, other values represent the differences between observed uptakes and the mean uninfected values at the respective temperature.

The results are shown in Table 2, which gives the thymidine uptake by uninfected controls and expresses the inhibition of uptake caused by \(hd+\), \(hd-5\), \(hd-19\), and the clones as the difference between the uptakes by the infected cultures and the average uptake by uninfected cultures. It can be seen that the ratio of the inhibition of uptake at 39.5°C to the inhibition of uptake at 37°C is similar for \(hd-19\) (2.7), \(hd+\) (2.0), and the clones (mean ratio = 1.88). Strain \(hd-5\) gives a normal inhibition at 37°C but is virtually completely defective in thymidine uptake inhibition at 39.5°C; none of the clones is at all similar to \(hd-5\). Independent assays at either temperature (fuller details of these findings will be presented elsewhere). A test for the ability to inhibit thymidine uptake at 39.5°C should therefore distinguish \(hd-5\) and \(hd-19\) genotypes in the mixed infection yields. Accordingly, 16 of the clones described above were tested for their effect upon thymidine uptake at 37.0°C and 39.5°C, as described in the Methods.

The results are shown in Table 2, which gives the thymidine uptake by uninfected controls and expresses the inhibition of uptake caused by \(hd+\), \(hd-5\), \(hd-19\), and the clones as the difference between the uptakes by the infected cultures and the average uptake by uninfected cultures. It can be seen that the ratio of the inhibition of uptake at 39.5°C to the inhibition of uptake at 37°C is similar for \(hd-19\) (2.7), \(hd+\) (2.0), and the clones (mean ratio = 1.88). Strain \(hd-5\) gives a normal inhibition at 37°C but is virtually completely defective in thymidine uptake inhibition at 39.5°C; none of the clones is at all similar to \(hd-5\). Independent assays
COMPLEMENTATION BETWEEN POLIOVIRUS MUTANTS

of the efficiencies of plating at 39.3° of these
clones gave values of 1 to 6 X 10^-5 (with
two exceptions of 10^-4, which contained some
wild-type plaques).

Thus although the clones were wild type
in thymidine uptake inhibition, they were
nevertheless highly heat defective, having
the plating efficiency of hd-19 (2 X 10^-5)
rather than that of hd-5 (10^-3). Furthermore,
hd-5 forms small plaques at 37.0°
whereas hd-19 plaques are wild type, and
few of the plaques in the mixed infection
yield could be regarded as typical of hd-5. It
is therefore concluded that the apparent
complementation observed was not as effec­
tive for one mutant as for the other and
that hd-19 predominated in the yield. The
number of clones tested was insufficient to
indicate that hd-5 was entirely excluded from
the yield. More extensive testing for this
point is likely to be inconclusive because of
the leakage of hd-5.

Effect of Time of Incubation on Yield En­

Figure 2 compares the one-step growth at
39.2° of hd-5 and hd-19, alone and in mixed
infection, with that of hd+. The conditions
were those of the standard complementa­
tion test described in the Methods, except
that tubes were removed and frozen at intervals for assay. The maximum en­
hancement in yield in mixed infections of
hd-5 and hd-19 occurred between 6 and 7
hours after infection, being 3 to 4 times the
sum of the individual mutant yields and
0.5 X 10^-3 of hd+ yields. The growth of the
mixed yield followed roughly the same time
course as the leak of both mutants (e.g.,
the 50% maximum yields all occurred at
the same time). However, growth of all
three mutants was delayed by almost one
hour when compared with that of hd+ under
the same conditions. All plaques in the 39.2°

Fig. 2. One-step growth at 39.2° of poliovirus strains hd+ (▲), hd-5 alone (○), hd-19 alone (◇), and
hd-5 plus hd-19 in mixed infection (⊙); the sum of the mutant yields when grown on their own is given
by ●. The hd+ curve is related to the left-hand ordinate, the others to the right. These five curves were
obtained by assay at 37°. The sixth curve (● ●) shows the assay at 39.2° of the hd-5 plus hd-19 mixed
infection cultures (for this assay, petri plates in sealed boxes were immersed in a water bath at 39.2°
for 3 days). The growth conditions were those of the complementation test (see Methods); replicate
tubes were removed at intervals and frozen for assay.
assay of the mixed yield (lowest curve) were small and indistinct, characteristic of mutant rather than $hd^+$; similar assays at $39.2^\circ$ of the growth cycles of each mutant alone gave plaques of similar number and character. Hence again (a) these growth curves of $hd$-5 and $hd$-19 by themselves represent leak at $39.2^\circ$ and not growth of contaminant wild type, and (b) less than $10^{-3}$ of the enhanced yield is wild type.

**Effect of Temperature on Yield Enhancement**

Several standard 6-hour complementation tests were performed concurrently in water baths at several different temperatures. Figure 3 shows that the excess of the mixed infection yields over the combined leak increased with the temperature over the range studied. At $38.4^\circ$ the mixed infection yield was less than twice the sum of the individual mutant yields, whereas at $39.6^\circ$ the mixed infection was 14 times the combined leaks. Hence enhancement of yield is easier to detect at the higher temperatures. Compared with wild-type yields, however, the enhanced yields (calculated as excess over combined leaks) decreased from $2 \times 10^{-3}$ to $3 \times 10^{-4}$ of $hd^+$ yield with increase in temperature from $38.4^\circ$ to $39.6^\circ$.

At temperatures above $39^\circ$ it is evident that the drop with increased temperature in leak, and presumably also in mixed infection yields, reflects some defect in the parental $hd^+$ or in the host cell as much as the defect peculiar to the mutant.

**Effect of pH on Yield Enhancement**

Four concurrent complementation tests done at pH values of 6.9, 7.1, 7.3, and 7.5, respectively, showed that the degree of enhancement was no greater at other pH values that covered the range optimal for growth of poliovirus. The mixed infection yields were 3.9, 4.3, 4.2, and 3.4 times the sum of individual mutant yields, and about

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**Fig. 3.** The effect of temperature on mixed infection of poliovirus strains $hd$-5 and $hd$-19. The procedure was that of the standard complementation test (see Methods); sets of tubes were incubated for 6 hours at the indicated temperatures. The points give the average yield of 4 replicate cultures of $hd$ alone (○) and $hd$-19 alone (△), and 8 replicate cultures of $hd$-5 plus $hd$-19 in mixed infection (●); ○ equals the sum of the mutant yields on their own, while △ equals $hd^+$ yield (right-hand ordinate).
Complementation Between Poliovirus Mutants

10^4 of that expected for hd+. The leak yields were also not much affected by pH.

Attempted Rescue of hd-5 and hd-19 by hd+

Previous sections of this paper indicate that the hd-5 and hd-19 stocks used contained few wild-type revertants. Accordingly, if wild-type enhances mixed infection yields with as low efficiency as the mutants, then the enhancement described above between hd-5 and hd-19 is most unlikely to result from some interaction between the mutants and traces of wild-type virus contained in the mutant stocks. The efficiency of enhancement with wild type was tested for by mixedly infecting cells with several high multiplicities of infection (4 to 20 PFU/cell) of either hd-5 or hd-19, together with hd+ in multiplicities from 10 PFU/cell to 1 PFU per 10^4 cells. At the lowest multiplicities the yield of hd+ when grown on its own was about the same as the mutant leak rates. The experimental procedure was that of the standard complementation test performed at 39.0°, and the 6.25 hour yields were assayed at 37° and 39.3°. The mutant hd-19 interfered markedly with hd+ investigations of this interference will be described elsewhere (Pohjanpelto and Cooper, 1965). However, in all cases any excess of the titre obtained by the 37° assay over that of the 39.3° assay (an excess presumably equal to the content of mutant virus) was no greater than the leak of mutant alone (2 × 10^3 PFU per 2 × 10^5 cells). It is therefore concluded that any rescue of mutant by wild-type virus is too small to be detected and hence cannot play a significant part in the reaction between hd-5 and hd-19 described above.

Discussion

These data show that hd-5 and hd-19 interact during growth at high temperatures; the reversion to wild type of both hd-5 and hd-19 is small but detectable, and this, together with the type of mutagen used to induce them (5-fluorouracil) and the type of defect (in which competent gene products are made at 37°) indicate that they contain point mutations rather than extensive changes.

Second, hd-5 and hd-19 may contain defects in the same gene, and interallelic complementation is usually of low efficiency. However, these mutants are defective in different physiological functions (for example, the inhibition of thymidine uptake described above); hence at least one of the mutants would have to contain two mutations, each leading to a gross defect at
39.2°, and one defect would have to be common to both mutants. Since crosses between hd-5 and hd-19 at 37° reproducibly yield enhanced yields of infective units that are negligibly defective at 39.2° (Cooper, unpublished), this possibility is also unlikely. Stronger evidence against interallelic complementation as the cause of the inefficient interaction between the hd mutants is that in no case was the enhancement of yield more efficient among over 100 “complementing” (i.e., yield-enhancing) pairs of hd mutants now studied (Cooper, unpublished); indeed the pair hd-5 and hd-19 was selected for further work for its relatively good performance. This study involved 31 different mutants that were isolated independently at a mutation rate of 10% (Cooper, 1964) or 2–3% and in which different physiological defects are often demonstrable (Cooper, Garves, and Johnson, unpublished).

Third, the low efficiency, like the inequality in the yields, may reflect the extensive interference which exists between hd and many of the hd mutants at high temperatures (Cooper, unpublished). Strain hd-19 happens to be a strong interfering agent, and the interference by hd-19 results from a hindrance to some event occurring after uncoating of the challenge virus and before its RNA synthesis (Pohjanpeltro and Cooper, 1965).

Fourth, possible effects of sequential gene function, coupled with sequestration of gene products or their dispersion through a large cell, and/or possible needs for some synchrony of gene function, might well reduce complementation efficiencies. The inefficient rescue of hd-5 by hd+ (a cis control of a kind) would imply some such case [hd-5 happens not to interfere with hd+ at high temperatures (Cooper, unpublished)].

REFERENCES