STRAIN DIFFERENTIATION OF
RUBELLA VIRUS

Thesis presented to the University of Surrey for the Degree of Doctor of Philosophy in the Department of Biological Sciences.

by

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Whilst the volume of literature concerning rubella virus has increased markedly during recent years, few authors have compared the properties of strains of varied origin and history. In the main, interest has centred on the biological characteristics of the attenuated strains of rubella and the modification of such properties during loss of virulence for man.

A study of the plaque morphology in GL-RK<sub>13</sub> cells, of some 36 strains from pre and post-natally acquired infection was undertaken. The strains included laboratory adapted, highly passaged and freshly isolated material with little or no history of culture in vitro. It was found that in the main, low pass strains showed a typical small plaque form but two freshly isolated strains with large plaque type were also encountered. The laboratory passaged strains showed a range in plaque morphology from small to large which did not appear to be dependant on the tissue selected for propagation of the virus. A brief study of the influence of tissue culture passage on the plaque morphology of selected strains yielded evidence of two systems of change; the first being a gradual transition from a small to large plaque, whilst the second appeared to be closer to a selection process, the new plaque type emerging and becoming predominant.

Six representative strains of rubella were selected for further study. Growth of virus and production of haemagglutinating antigen in several cell culture systems was compared and the results suggested that the previous passage history of a strain could influence its potential for growth in cell culture.

The thermal stability of these strains was also investigated and the two isolates from congenitally infected infants were shown to differ in their pattern of inactivation.
All of the strains were shown to be immunogenic in rabbits, and the antisera thus produced were used in cross haemagglutination inhibition and neutralization studies. Apparent anomalies were encountered with the antisera produced in response to the Cendehill strain. It was not possible to demonstrate haemagglutination inhibiting antibodies against some of the strains in spite of the fact that these were present to high titres against other strains of rubella. Examination of the kinetics of homologous and heterologous neutralization showed some difference between the strains and the existence of two particularly antibody sensitive strains.
I would like to offer my sincere thanks to my supervisors Miss Gwen Laurence, Dr. Michael Butler and Dr. Stanley Piercy for their excellent advice and unfailing patience. My thanks go to Dr. June Almeida for her help during the final stages of this thesis.

I also express my gratitude to Mrs. Joyce Dains for her long hours at the typewriter and the Photographic Department of the Wellcome Research Laboratories for the Figures included in this work.

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"Please do not shoot the pianist. He is doing his best"

Oscar Wilde.

Impressions of America "Leadville"
Introduction

Section I
Assay of viral infectivity by the plaque technique in GL-RK, cells

Section II
The cytopathic effects of rubella virus strains in selected tissues.

Section III
The growth characteristics of selected strains of virus in varied culture systems.

Section IV
The thermal stability of selected strains of rubella virus as measured by surviving infectivity.

Section V
Immunogenicity of rubella virus in rabbits and a comparison of selected strains by haemagglutination inhibition and neutralization tests.

Discussion

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Abbreviations
First described as Rوطه as Rوطه, as early as 1866, controversy continued as to its existence as an entity for a further fifteen years. In 1881 the International Congress of Medicine in London gave the official seal of recognition to Rubella, acknowledging it as a disease distinct from Measles or Scarlet Fever. Little further progress occurred until 1938, when Hiro and Tasaka were able to demonstrate the viral etiology of the infection by transmission of the disease with filtered throat washings from infected individuals. Rubella was generally regarded as a trivial infection with little clinical importance until 1941, when Sir Norman McAllister Gregg published his observations on the apparent relationship between rubella infection during pregnancy and congenital defects in the offspring. Although Gregg's report was supported by those of Swan and his colleagues in 1943 and 1944, the full importance of his observations were not realised until 1947 when Wesselhoeft published a comprehensive account of rubella emphasising its dramatic consequences to the pregnant woman.

The virus eluded isolation until 1962 when simultaneous papers from the Walter Reed Army Institute of Research (Parkman, Buescher and Artenstein, 1962) and the Harvard School of Public Health (Weller and Neva, 1962), reported the recognition of rubella virus in culture by either the failure to superinfect African Green Monkey kidney cells with Echo virus type 11, or the slow evolution of a cytopathic effect in primary human amnion cells. Recent years have brought major advances in the investigation of rubella virus. It has been shown to be a rubonucleic acid virus of some 70-75 nanometres, with a pleomorphic structure exhibiting an internal, electron dense, core of 35 nanometres and a roughened outer surface suggestive of projections (Best, Banatvala
It is thought to contain protein and lipid due to its buoyant density of 1.18 gm/ml, its sensitivity to lipid solvents and the lytic action of complement. (Liebhaber et al, 1969; Almeida and Laurence, 1969). Ribonucleic acid with a molecular weight of $3 \times 10^6$ daltons, may be extracted from the virus and has been shown to retain its infectivity for tissue culture (Hovi and Vaheri, 1970; Sedwick and Sokol, 1970).

Haemagglutinin, complement fixing antigen and two precipitating antigens have been demonstrated for rubella virus. The haemagglutinin, first described by Stewart, Parkman, Hopps, Douglas, Hamilton and Meyer in 1967, has been shown to be largely associated with the complete virus, (McCombs and Rawls, 1968) although it is most usually employed as an ether-detergent degraded subunit for antibody estimations, as this process yields higher titre material. Two complement-fixing antigens have been recognised (Schmidt and Lennette, 1969), the first of high molecular weight is the so-called insoluble antigen which is always associated with infectivity and haemagglutinin, whilst the second soluble antigen, elutes separately from a fractionation column. It is thought that the small soluble antigen may well represent a subunit of the virus, but it is as yet unclear as to whether it is a degradation product or excess material which might be incorporated into the complete virion. The two precipitating antigens theta and iota, (Le Bouvier, 1969) are also thought to represent structural components of the virus.

The classification of rubella virus is not as yet finally decided, although similarities have been noted to the Group A arboviruses (Holmes, Wark and Warburton, 1969), Bittner mouse mammary tumour agent, (Almeida et al, 1969) and the avian leukosis group, (Horstmann, 1971). It is at present placed in the Togaviridae due to morphological and biological similarities to other Togaviruses, but remains ungrouped. (Horzinek, Maess and Laufs, 1971).
Advances in the characterisation of Rubella virus led to interest in the possibility of vaccines intended for the protection of the unborn child. Three strains of the virus have been attenuated and are now available, the HPV-77 strain, (Parkman, Meyer, Kirschstein and Hopps, 1966) the Cendehill strain (Huygelen, Peetermans and Prinzie, 1969) and the RA27/3 strain (Plotkin, 1969). These attenuated strains of rubella have been shown to have properties which differed from the origin material, (Parkman et al, 1966; Huygelen et al, 1969; Plotkin et al, 1969) and which were linked with loss of virulence for man. Meyer, Parkman, Hoesins, Larson, Davis and Simsarian, (1969) have suggested that suitable markers of attenuation of rubella virus may be found in a) cytopathogenicity and plaque formation in GL-RK, cells b) levels of interferon induced in culture, and c) virus shedding and evidence of viraemia in infected monkeys. Similar changes in property have been demonstrated for other strains of rubella virus on passage; these may not necessarily be related to attenuation, for example, the increased cytopathic effect of Judith strain in GL-RK, cells after passage in this tissue (Morgan, 1969; Laurence and Gould, 1969). It was interesting to note that the three vaccine strains of rubella virus have few laboratory markers of attenuation in common. It was not known whether the variation in the laboratory properties of these vaccine strains was due to the different tissues and methods employed in the attenuation process or to a basic difference in the original isolates.

Rubella has posed interesting epidemiological problems. It is a disease normally occurring in late childhood and early adolescence, however this pattern has been found to vary in certain countries, with high levels of immunity being reached by the age of five in some South American communities, whilst in other areas of the world low numbers of immune subjects are found at all ages in spite of endemic rubella (Horstmann, 1971). Furthermore it has been noted that Japan has a
maternal rubella whilst still retaining endemic rubella in the community and an expected percentage of susceptible subjects, (Kono, 1969). The incidence of widespread epidemics appears to be cyclic in countries such as the USA, occurring every six to nine years with a steady resurgence of the diseases every spring in the intervening years, (Horstmann, 1971); however, figures from Australia have suggested that a sizeable epidemic occurs yearly (Forbes, 1969).

Seigal, Fuerst and Guinea (1971) have suggested that the abnormal pandemic which occurred in the USA in 1964 with its attendant rise in the number and pattern of congenital defects may well be explained by a biological change in the circulating virus. Horstmann (1971) similarly suggested that the unusual epidemiological behaviour of rubella might be explained by such factors but recognised that no significant variation in rubella virus strains had been demonstrated as yet. The availability of vaccine together with the increasing number of questions associated with the epidemiology and properties of rubella virus in the community, naturally led to a growing interest in the possibility of biological variation in rubella virus. Simple cross-neutralisation and haemagglutination inhibition studies, (Kono, 1969; Banatvala and Best, 1969) or the more sophisticated kinetic haemagglutination-inhibition studies of Best and Banatvala in 1970, did not reveal a significant antigenic difference between the strains selected for study. Not even strains exhibiting an apparent variation in one biological property, such as teratogenic potential for the foetus, could be shown to vary antigenically (Kono, 1969). Fogel and Plotkin (1969) and Oxford (1969) however, were able to demonstrate some small antigenic variation between strains in simple neutralisation tests although the values obtained lay near to the level of significance. Several workers had reported that highly passaged strains could be distinguished from those with little
cytopathogenicity in GL-RK₁₃ or BHK21/13S cells, (Parkman et al., 1966; Fogel et al., 1969; Morgan, 1969, Oxford, 1969; Plotkin, 1969), levels of interferon induced in culture (Parkman et al., 1969; Huygelen et al., 1969) and immunogenicity for monkeys and rabbits, (Parkman et al., 1966; Huygelen et al., 1969; Oxford, 1969) but few comparisons were made of strains with similar or low passage histories. Banatvala, Potter and Best (1971), however were able to demonstrate a significant difference in the interferon induced in culture by one low passage strain, KO-1, when compared to strains of similar history and to highly passaged attenuated strains.

It was decided that a comparison of certain in vitro and in vivo properties of strains of rubella virus from varied sources, with similar passage histories together with the three attenuated vaccine strains might prove interesting. Certainly some variation in biological properties between the strains had already been demonstrated, plaque morphology in GL-RK₁₃ cells being the most well documented, but it was not known how such properties could be influenced by differences in the cultural histories of these strains, thus a short investigation was carried out on the influence of passage. A comparison of cytopathogenicity in GL-RK₁₃ cells, together with several other cell systems in common use was undertaken, as was a survey of the thermal stability of selected strains, for Kistler and Sapatino (1972) had suggested that the apparently low sensitivity of rubella to high temperatures might be a function of adaptation of the virus and might therefore differ between strains of varied history. Huygelen et al (1969) and Oxford (1969) had presented evidence to show differences in the immunogenicity of strains of rubella virus in rabbits; this property was compared for the three vaccine strains and for low pass strains of similar history. The antisera yielded by such experiments were then employed for simple comparisons of the antigens.
variation has been demonstrated for rubella virus, however, the more sensitive techniques such as kinetic neutralization (McBride, 1959) had not been employed, therefore a comparison by this method was undertaken in this project. Comparisons of plaque morphology in GL-RK_{13} cells, estimation of growth curves, thermal stability, and neutralization indices required a reliable and reproducible plaque test of good sensitivity, thus an investigation of the factors influencing the GL-RK_{13} plaque test system together with a determination of the optimum conditions of operation were undertaken.
SECTION I

Assay of Viral Infectivity by the plaque technique in GL-RK₁₃ cells
Introduction

Materials and Methods

1) Cell and Tissue Cultures

2) Virus Strains

3) Isolation of rubella virus from clinical specimens

4) Production of pools of the standard control rubella virus strains

5) Production of working stocks of selected strains of rubella virus

6) Standard rubella antisera

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   B) Modified method

8) Direct infectivity assay in GL-RK_{13} cells in microtitre trays

9) Indirect infectivity assay in primary monkey kidney tissue by the Interference technique

10) Treatment of sera for use in overlay medium

Results

1) Investigation of factors influencing plaque formation
   A) Source of GL-RK_{13} cells
   B) Growth conditions for stock cultures and monolayers for infection
      i) Serum incorporated in the medium
      ii) Cell seeding rate
      iii) Incubation conditions
   C) Conditions for infection and maintenance of the monolayers
      i) Washing of monolayers
      ii) Volume of inoculum
      iii) Adsorption time
iv) Overlay medium
   (a) Serum
   (b) Sodium bicarbonate
   (c) Agar

v) Incubation conditions

2) Sensitivity of the modified test as a titration system
   (A) Dose-response relationship
   (B) Specificity of plaques
   (C) Reproducibility of the test
      (i) Variation of the plaque count
      (ii) Frequency distribution of plaque counts within one test and consecutive tests
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3) Methods of measurement of the diameter of plaques and the reproducibility of the values obtained

Discussion
rubella virus produced a cytopathic effect in GL-RK\textsubscript{13} cells which led directly to the application of this cell system by Taylor-Robinson, McCarthy, Grylls and O'Ryan (1964) to two plaque techniques; the first giving macroplaques at 5 to 7 days under an agar overlay and the second giving microplaques at 50-60 hours under a fluid medium. The microplaque technique was lengthy and necessitated a count of all the foci in a culture under low power magnification whilst the macroplaque method could be counted quickly by the naked eye after removal of the overlay and staining of the cell sheet. Both techniques were found to be reliable in the experienced hands of the original authors, but others have found it necessary to modify the original technique in order to obtain reproducible results. For example, Plotkin (1965) found that optimum results were obtained if the cell sheet were stained with neutral red in a second overlay introduced at the 7th to 8th day, and that lactalbumen hydrolysate in the overlay in place of 199 and calf serum, yielded equally good results. Fogel et al (1969) showed that higher plaque counts and more uniform plaque morphology could be achieved if agarose or carboxy methyl cellulose replaced agar as the solidifying agent. Hekker, Huisman and Jongeling (1969) reported optimum results under agar overlays containing 199, calf serum and skimmed milk, the latter being incorporated to enhance the clearness of the plaques when the cell sheet was stained.

Few other reports of the factors affecting rubella plaque formation in GL-RK\textsubscript{13} cells appear in the literature, Taylor-Robinson et al (1964) stated that the precise appearance of the plaques could be influenced by the strain of GL-RK\textsubscript{13} cells used, the incubation time and the density of the monolayer. Hekker et al (1969) found that in their plaque technique the concentration of skimmed milk and sodium bicarbonate were critical for plaque formation and that once an optimum
in order to ensure the reproducibility of the results. In this laboratory the original method of Taylor-Robinson et al (1964) was investigated using the standard control viruses Sl (RA27/3) and ARl (HPV-77). A modified technique was developed which produced a system for the comparison of the plaque characteristics of different strains of rubella virus and a reliable infectivity assay.
1) **Cell and Tissue cultures**

The cell systems utilized in this investigation are listed in Tables 1(a) and 1(b). Details of the source, conditions of subculture, growth and maintenance requirements are included. All media and sera were supplied by Wellcome Reagents Ltd., except foetal calf serum which was obtained from Flow Laboratories Ltd. All media contained 200 units/ml of penicillin and 100 units/ml of streptomycin. Incubation was carried out at 36.5°C throughout unless otherwise stated.

Full details of each medium constituent and the equipment used in this study are given in the Appendix.

2) **Virus strains**

(a) **Rubella virus.**

The strains of rubella virus included in this study are listed in Tables 2(a), (b) and (c) with details of their origin. All material was stored at -70°C unless otherwise stated. Samples received from other laboratories were immediately stored at -70°C until they could be examined in a GL-RK\textsubscript{13} plaque test, at which time the material was broken down to 0.1ml volumes for further storage at -70°C, or passaged through GL-RK\textsubscript{13} cultures to yield a secondary pool. Strains selected for more detailed examination were typed as rubella virus against standard hyperimmune antiserum to the Edmund strain of rubella, or human rubella convalescent serum.

(b) **M6 Virus**

Pools of bovine enterovirus M6 (Draper and Laurence, 1969) were produced in primary Erythrocebus Patas monkey kidney tissue cultures. Samples of each pool were titrated in the same tissue type and the material held in 0.5ml aliquots at -20°C.
<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>SOURCE</th>
<th>GROWTH MEDIUM</th>
<th>MAINTENANCE MEDIUM</th>
<th>SUBCULTIVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL-RK&lt;sub&gt;13&lt;/sub&gt;</td>
<td>a) National Institute of Health, USA</td>
<td>a) Eagles MEM, 0.11% NaHCO₃, 5% Adult bovine serum</td>
<td>Eagles MEM, 0.22% NaHCO₃, 2% Foetal or agamma calf serum</td>
<td>1 - 3 days</td>
</tr>
<tr>
<td></td>
<td>b) LRC</td>
<td>b) as above</td>
<td></td>
<td>7 day intervals</td>
</tr>
<tr>
<td></td>
<td>c) CTR</td>
<td>c) Eagles MEM, 0.11% NaHCO₃, 5% Foetal calf serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VERO</td>
<td>Medical Research Council, Colindale, UK</td>
<td>Eagles MEM, 0.11% NaHCO₃, 5% Foetal calf serum</td>
<td>Eagles MEM, 0.22% NaHCO₃, 2% Foetal or agamma calf serum</td>
<td>1 - 4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>or Eagles MEM, 0.22% NaHCO₃, 2% glucose</td>
<td>3/4 days intervals</td>
</tr>
<tr>
<td>BHK21/13S</td>
<td>Wellcome Research Unit, Pirbright, UK</td>
<td>Eagles MEM, 0.11% NaHCO₃, 10% tryptose phosphate broth, 5% adult bovine serum</td>
<td>Eagles MEM, 0.22% NaHCO₃, 10% tryptose phosphate broth, 2% Foetal or agamma calf serum</td>
<td>1 - 8 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>or Eagles MEM, 0.22% NaHCO₃, 2% glucose</td>
<td>3/4 days intervals</td>
</tr>
<tr>
<td>WI-38</td>
<td>Medical Research Council, Hampstead, UK</td>
<td>Eagles BME, 0.07% NaHCO₃, 10% Foetal calf serum</td>
<td>Eagles BME, 0.11% NaHCO₃, 2% Foetal calf serum</td>
<td>1 - 2 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3/4 days intervals</td>
</tr>
<tr>
<td>McCoy</td>
<td>Institute of Ophthalmology, London, UK</td>
<td>Eagles MEM, 0.11% NaHCO₃, 10% Foetal or agamma calf serum</td>
<td>Eagles MEM, 0.22% NaHCO₃, 2% Foetal or agamma calf serum</td>
<td>1 - 4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3/4 days intervals</td>
</tr>
</tbody>
</table>

Table 1 (a) Continuous Cell Lines
<table>
<thead>
<tr>
<th>TISSUE CULTURE</th>
<th>SOURCE</th>
<th>GROWTH MEDIUM</th>
<th>MAINTENANCE MEDIUM</th>
<th>SEEDING RATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young Rabbit</td>
<td>6 week old, Californian strain rabbits</td>
<td>Earles B.S.S., 0.22% NaHCO₃, 0.5% lactalbumin hydrolysate, 2.5% Foetal calf serum</td>
<td>199, 0.11% NaHCO₃, 0.5% lactalbumin hydrolysate, 10% liver digest, 1% Foetal calf serum, 0.001 M Arginine, 0.001 M Glycine</td>
<td>2.5 x 10⁴ cells per sq.cm. conflucent in 6-7 days</td>
</tr>
<tr>
<td>Erythrocebus</td>
<td>Quarantined monkeys, received weekly at the Wellcome Research Laboratories</td>
<td>Earles B.S.S., 0.11% NaHCO₃, 0.5% lactalbumin hydrolysate, 2.5% adult bovine serum</td>
<td>Earles B.S.S., 0.22% NaHCO₃, 0.5% lactalbumin hydrolysate, 5.0% liver digest, 2% bovine serum albumin</td>
<td>5.0 x 10⁴ cells per sq.cm. conflucent in 6-7 days</td>
</tr>
<tr>
<td>Chick Embryo</td>
<td>Specific pathogen-free eggs, supplied from T.A.D., Germany used at 9-11 days.</td>
<td>Earles B.S.S., 0.11% NaHCO₃, 0.5% lactalbumin hydrolysate, 5% Foetal calf serum</td>
<td>199, 0.22% NaHCO₃, 1% Foetal calf serum</td>
<td>4.0 x 10⁵ cells per sq.cm. 24-36 hours</td>
</tr>
</tbody>
</table>

Table 1 (b) Primary Tissue Cultures.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ORIGIN</th>
<th>HISTORY ON RECEIPT</th>
<th>OBTAINED FROM:-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isolated from natural infections of adults and children</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAY</td>
<td>Throat swab</td>
<td>3rd pass in GL-RK&lt;sub&gt;13&lt;/sub&gt;</td>
<td>) Professor Dudgeon,</td>
</tr>
<tr>
<td>SHEPPARD *</td>
<td>Throat swab</td>
<td>6th pass in GL-RK&lt;sub&gt;13&lt;/sub&gt;</td>
<td>) Gt. Ormond Street Hospital for</td>
</tr>
<tr>
<td>JUDITH (A)</td>
<td>Lymph node</td>
<td>3rd pass in primary rabbit kidney</td>
<td>) Sick Children, London. UK.</td>
</tr>
<tr>
<td>LESLEY *</td>
<td>Throat &amp; nasal swab</td>
<td></td>
<td>) Professor McCarthy, University</td>
</tr>
<tr>
<td>JANINE *</td>
<td>Throat &amp; nasal swab</td>
<td>Fresh clinical specimen</td>
<td>) of Liverpool, UK.</td>
</tr>
<tr>
<td>ROBB</td>
<td>Throat &amp; nasal swab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BROWN</td>
<td>Throat swab</td>
<td>5th pass in primary monkey kidney</td>
<td>) Dr. Fucillo, National Institute</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>) of Neurological Diseases</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>) and Blindness, U.S.A.</td>
</tr>
<tr>
<td><strong>Isolated from vaccinees</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.S. (CENDEHILL)</td>
<td>Throat swab</td>
<td></td>
<td>)</td>
</tr>
<tr>
<td>GEL (HPV-DUCK)</td>
<td>Throat swab</td>
<td>1st pass in GL-RK&lt;sub&gt;13&lt;/sub&gt;</td>
<td>) Professor Dudgeon</td>
</tr>
<tr>
<td>DOU (RA27/3)</td>
<td>Throat swab</td>
<td></td>
<td>)</td>
</tr>
<tr>
<td>GEDDES (RA27/3)</td>
<td>Throat swab</td>
<td>Fresh clinical specimen</td>
<td>)</td>
</tr>
<tr>
<td>McNULTY (RA27/3)</td>
<td>Throat swab</td>
<td></td>
<td>)</td>
</tr>
<tr>
<td><strong>Isolated from experimentally infected Erythrocebus patas monkeys</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK 6 (HPV-77)</td>
<td>Nose swab</td>
<td>1st pass in primary monkey kidney</td>
<td>)</td>
</tr>
<tr>
<td>MK 10 (SHEPPARD)</td>
<td></td>
<td></td>
<td>)</td>
</tr>
</tbody>
</table>

* Typed as rubella, with specific antiserum.

Table 2(a) Low pass strains of rubella virus isolated from postnatally acquired infections.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ORIGIN</th>
<th>HISTORY ON RECEIPT</th>
<th>OBTAINED FROM:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated from foetal tissues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H.E.</td>
<td>Foetal kidney</td>
<td>1st pass in GL-RK\textsubscript{13}</td>
<td>Dr. Banatvala, St. Thomas Hospital</td>
</tr>
<tr>
<td>WRIGHT</td>
<td>Foetal liver</td>
<td>3rd pass in GL-RK\textsubscript{13}</td>
<td>London, U.K.</td>
</tr>
<tr>
<td>SAVVA</td>
<td>Placenta</td>
<td>Fresh clinical specimen</td>
<td></td>
</tr>
<tr>
<td>Isolated from rubella syndrome infants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMBERT</td>
<td>Unknown</td>
<td>1st pass in GL-RK\textsubscript{13}</td>
<td>Dr. Banatvala</td>
</tr>
<tr>
<td>FULLER</td>
<td>Throat swab</td>
<td>2nd pass in GL-RK\textsubscript{13}</td>
<td>Professor Dudgeon, Gt. Ormond Street Hospital for Sick Children, London, U.K</td>
</tr>
<tr>
<td>ANDREWS</td>
<td>Throat swab</td>
<td>3rd pass in GL-RK\textsubscript{13}</td>
<td>Dr. Banatvala</td>
</tr>
<tr>
<td>THOMAS (E) *</td>
<td>Lens tissue</td>
<td>3rd pass in GL-RK\textsubscript{13}</td>
<td>Professor Dudgeon</td>
</tr>
<tr>
<td>DUNNING *</td>
<td>Throat swab</td>
<td>4th pass in GL-RK\textsubscript{13}</td>
<td></td>
</tr>
<tr>
<td>THOMAS (T)</td>
<td>Throat swab</td>
<td>7th pass in GL-RK\textsubscript{13}</td>
<td></td>
</tr>
<tr>
<td>SIMONI</td>
<td></td>
<td>2nd pass in monkey kidney tissue</td>
<td></td>
</tr>
<tr>
<td>HITCHCOCK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIGUERRE (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CARNWRIGHT</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEFEBVRE (1)</td>
<td></td>
<td>3rd pass in monkey kidney tissue</td>
<td></td>
</tr>
<tr>
<td>GABRIEL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOLDTHORPE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEFEBVRE (2)</td>
<td></td>
<td>6th pass in monkey kidney tissue</td>
<td></td>
</tr>
</tbody>
</table>

* Typed as rubella with specific antiserum.

Table 2 (b) Low pass strains of rubella virus isolated after intra-uterine infections
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ORIGIN</th>
<th>HISTORY ON RECEIPT</th>
<th>OBTAINED FROM:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated from post-natal infections of adults and children</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JUDITH (B)</td>
<td>Throat swab</td>
<td>50&lt;sup&gt;th&lt;/sup&gt; pass in GL-RK&lt;sub&gt;13&lt;/sub&gt;</td>
<td>)</td>
</tr>
<tr>
<td>CENDEHILL*</td>
<td>Throat swab</td>
<td>51&lt;sup&gt;st&lt;/sup&gt; pass in primary rabbit kidney</td>
<td>)</td>
</tr>
<tr>
<td>HPV-77*</td>
<td></td>
<td>76&lt;sup&gt;th&lt;/sup&gt; pass in primary monkey kidney</td>
<td>Miss G.D. Laurence</td>
</tr>
<tr>
<td>HPV-77 (YRK)</td>
<td>Throat swab</td>
<td>78&lt;sup&gt;th&lt;/sup&gt; pass in primary monkey kidney</td>
<td>Wellcome Research Laboratories</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ 3&lt;sup&gt;rd&lt;/sup&gt; pass in primary rabbit kidney</td>
<td>U.K.</td>
</tr>
<tr>
<td>HPV-150+12 CETO</td>
<td></td>
<td>150&lt;sup&gt;th&lt;/sup&gt; pass in primary monkey kidney</td>
<td>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ 12&lt;sup&gt;th&lt;/sup&gt; pass in chick embryo tissue</td>
<td>)</td>
</tr>
<tr>
<td>Isolated from intra-uterine infections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIGUERRE (2)</td>
<td>Unknown</td>
<td>2nd pass in monkey kidney + 7th pass in GL-RK&lt;sub&gt;13&lt;/sub&gt;</td>
<td>Dr. Banatvala, St.Thomas Hospital, London. U.K.</td>
</tr>
<tr>
<td>RA27/3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Foetal kidney</td>
<td>4&lt;sup&gt;th&lt;/sup&gt; pass in human kidney fibroblasts + 30&lt;sup&gt;th&lt;/sup&gt; pass in WI-38</td>
<td>Dr. S.A. Plotkin, Wistar Institute. U.S.A.</td>
</tr>
</tbody>
</table>

* Typed as rubella with specific antiserum

Table 2 (c) Laboratory adapted strains of rubella virus with greater than seven passages in any tissue.
Isolation from throat or nasal swabs:

The swab was immediately placed in 1 ml. of transport medium, which consisted of Eagles BME, 0.13% NaHCO₂, double strength antibiotics and 5% Solupro (degraded gelatin stabilizer), and was either cultured immediately or stored at -70°C until cell cultures were available.

Swabs were thoroughly squeezed out into the transport medium and 0.2 ml. aliquots were inoculated into 2 or 3 tube cultures of primary monkey kidney and GL-RK₁₃ cells. Where possible 0.1 ml. volumes were also inoculated onto confluent GL-RK₁₃ plates which were then overlaid in the normal manner (see later). Monkey kidney cultures were harvested at 10 days and then challenged with No bovine enterovirus in order to detect the presence of rubella. GL-RK₁₃ cultures were examined daily from 5 - 14 days post inoculation for the appearance of cpe, positive cultures were harvested when the cpe was maximum, whilst negative cultures were harvested at the end of the observation period. GL-RK₁₃ plates were fixed and stained at 7 days and examined for the presence of plaques. Samples apparently negative on the first passage were subjected to a further two sub-inoculations to confirm the absence of rubella virus.

Isolation from nasal washings

1.0 ml. of 0.85% Saline was introduced into each nostril of the subject and the effluent collected in a small enamel bowl. 1 ml. of these washings was added to 1.0 ml. of double strength transport medium, and handled in the manner described above, (Moffat, Gould, Forbes, Freestone and Macdonald, 1972).

Isolation from tissue

Small pieces of selected tissue were finely minced in 1.0 ml. volumes of transport medium without Solupro stabilizer. The samples were subjected to 3 cycles of freezing and thawing and clarified by
Solupro stabilizer at a final concentration of 5\% was added for storage and the samples were treated as described above.

4) Production of pools of the standard control rubella virus strains

AR/1/1B; HPV 77 strain received from Dr. H. Meyer of the Division of Biological Standards, passaged at the Wellcome Research Laboratories in Erythrocebus patas monkey kidney cultures. Virus harvested after two passages was stabilised with sorbitol and degraded gelatin and freeze dried.

SI; RA27/3 strain received from Dr. S. Plotkin of the Wistar Institute at the 25th passage level was passaged in WI-38 cell cultures at the Wellcome Research Laboratories. Virus harvested after four passages was stabilised and freeze dried.

5) Production of stocks of selected strains of rubella virus

The 6 strains selected for detailed study were HPV-77, RA27/3, Cendehill, Dunning, Thomas and Lesley. Working pools of these strains were produced in Vero cultures, (Liebhaber, Riordan and Horstmann, 1967). Roux bottles of Vero cells were drained of medium at 3 to 4 days old and were washed 3 times with serum-free maintenance medium. Each of 5 bottles received 5 ml. inoculum of the chosen strain at a passage level expected to replicate sufficiently well to give adequate infectivity titres (see Section III). The inoculum was allowed to adsorb for 30 minutes at 36.5\(^\circ\)C before the cultures were refed with serum-free medium, and a further change was carried out at 24 hours to ensure that little serum remained in the culture fluid. At 5 days post-infection, the fluid was harvested, pooled from the five vessels and dispensed in 2 ml. volumes which were stored at -70\(^\circ\)C. The cells from infected cultures were scraped into 2 ml. of fluid harvest, an equal volume of glycine buffer at pH 10 was added and the whole was set at 36.5\(^\circ\)C for 18 to 24 hours to allow extraction of the antigen. The cell suspensions were
CO₂ bath, pooled and spun lightly at 800 rpm on an M.S.E. Minor centrifuge to remove gross cellular debris. The supernatant was stored in 0.1 ml. volumes and held at -20°C. Aliquots of fluid harvests were titrated for infectivity by the plaque test and samples of the alkaline extract were assayed for haemagglutinin content. On some occasions the haemagglutinin or virus titre was not considered adequate and further cultures were infected with the harvested material and the process repeated. The passage level at which each pool was produced is listed in Table 3, together with details of the previous history and titre of each strain.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>PREVIOUS HISTORY</th>
<th>PASSAGE LEVEL IN VERO + INFECTIVITY</th>
<th>TITRE AT WHICH POOL WAS LAID DOWN HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA27/3</td>
<td>PASS 30 in WI-38</td>
<td>PASS 2 at 5.45 pfu/0.5 ml</td>
<td>PASS 3 at 64 HAU</td>
</tr>
<tr>
<td>HPV-77</td>
<td>PASS 78 in Monkey kidney tissue</td>
<td>PASS 5 at 6.20 pfu/0.5 ml</td>
<td>PASS 5 at 128 HAU</td>
</tr>
<tr>
<td>CONDEHILL</td>
<td>PASS 53 in YRK</td>
<td>PASS 3 at 6.50 pfu/0.5 ml</td>
<td>PASS 4 at 64 HAU</td>
</tr>
<tr>
<td>DUNNING</td>
<td>PASS 6 in GL-RK$^{13}$</td>
<td>PASS 3 at 4.65 pfu/0.5 ml</td>
<td>PASS 4 at 32/64 HAU</td>
</tr>
<tr>
<td>THOMAS</td>
<td>PASS 3 in GL-RK$^{13}$</td>
<td>PASS 2 at 4.68 pfu/0.5 ml</td>
<td>PASS 3 at 128 HAU</td>
</tr>
<tr>
<td>LESLEY</td>
<td>PASS 1 in Monkey kidney tissue</td>
<td>PASS 2 at 4.40 pfu/0.5 ml</td>
<td>PASS 4 at 32/64 HAU</td>
</tr>
</tbody>
</table>

**TABLE 3.** Passage history and titre of pools of rubella virus
6) Standard rubella antisera

Antiserum R50/70:

A rabbit of the Californian strain received a course of inoculations given at 14 day intervals into the ear vein. The inoculum consisted of 5 ml of the Edmund strain of rubella virus (10^4.5 pfu/ml) grown in GL-RK_13 cell cultures. Fourteen days after the final inoculation the rabbit was bled out, the serum separated and stored in 1.0 ml volumes at -20°C.

Antiserum J27/3

This serum was collected from a female adult volunteer 12 months after natural rubella. 300 ml of whole blood was taken, the serum separated, dispensed into 1.0 ml volumes and stored at -70°C.

7) Plaque test for rubella virus in GL-RK_13 cells

A) Original method

Stock cultures of GL-RK_13 (CTR) were grown and subcultured as indicated in Table 1 (a). 60 mm. plastic dishes (Falcon) were seeded with sufficient cells to become confluent in 2 to 3 days, these were normally in the order of 2.0 x 10^6 cells in 10 ml. The confluent cultures were drained and washed once prior to infection with buffered Eagles MEM which was also used as diluent for viral titrations. Adsorption of the 0.2 ml. inoculum was allowed for 45 minutes at room temperature before introduction of 10 ml of overlay consisting of Eagles MEM, 0.089% NaHCO_3, 2% foetal calf serum and 1% Difco Noble Agar. The overlay was allowed to solidify before the cultures were returned to incubation at 36.5°C in an atmosphere of 5% CO_2 in air. Sample plates were removed from 5 to 8 days post infection and stained with crystal violet in formalized phosphate buffered saline after removal of the overlay. All the plates were stained when the plaques were thought to be optimum in the sample culture which was usually 6 or 7 days post
made from 3 plates at each dilution and expressed as pfu/0.5ml.

B) Modified Method

The test was carried out in 35 or 60 mm Falcon plastic petri dishes, seeded with sufficient cells to form a confluent monolayer in 72 hours, this was normally in the order of 1 x 10^6 cells for a 35 mm dish or 1.5 - 2.0 x 10^6 cells for a 60 mm dish contained in 3 and 8 ml. of growth medium respectively. The cultures were incubated in a modified LTE cabinet at a temperature of 32.5°C and a humidified atmosphere of 5% CO_2 in air, thus maintaining a pH of 7.2 - 7.4.

Confluent plates were drained and washed once with buffered Eagles MEM, such medium also being used as virus diluent. The inoculum was introduced onto the centre of the dish in volumes of 0.1 ml. for a 35mm, and 0.2 ml for a 60 mm dish. Adsorption was allowed for 20-30 minutes at room temperature, before the overlay, held at a temperature of 50-60°C, was introduced, the required volumes being 4 and 8 ml. for 35 mm and 60 mm dishes respectively. The overlay medium consisted of Eagles MEM, 0.088% NaHCO_3, 2% foetal or agamma calf serum, and 1% Difco Noble Agar. The plates were left at room temperature for 15-20 minutes before being returned to the cabinet, in order that the overlay might solidify before the dishes were moved. The infected cultures were incubated for 7 days, at which time the overlay was removed and the cell sheets were fixed and stained with crystal violet in formalized phosphate buffered saline.

Measurement of plaque diameters was carried out with the aid of a transparent rule over a glass topped box, evenly illuminated from below by a fluorescent light tube source. Samples of the standard pools of HPV-77 (AR1) and RA27/3 (S1) were included in every test, to serve as a control for the reproducibility of plaque sizes and infectivity assays obtained in the test system.
occasions in separate plaque tests, and when one strain was compared
to another they were always both examined on at least one occasion in one
plaque test. The plaque sizes quoted were based on the values obtained
from a minimum of 3 plates in any one test.

8) Direct Infectivity assay in GL-RK<sub>13</sub> cells in microtitre trays

Dilutions of virus were made in Eagles MEM growth medium containing
5% foetal or agamma calf serum + 0.1% NaHCO<sub>3</sub>. 0.5ml volumes of each
dilution were added to 1 ml. aliquots of a suspension of GL-RK<sub>13</sub> cells
at a concentration of 2.5 x 10<sup>5</sup> cells/ml. in the same medium. The
samples were mixed thoroughly and 0.1 ml. aliquots of each dilution were
added to each of 8 cups in a sterile Falcon plastic microtitre tray.
An uninoculated control of medium and cells was also included. The trays
were covered with loose fitting plastic lids or with gas-permeable sealing
taps and incubated at 32.5°C in a CO<sub>2</sub> incubator. The cultures were read
on alternate days up to 14 days post inoculation using an inverted
microscope. Virus titres were calculated by the method of Karber (1931)
and expressed as TCD<sub>50</sub>/0.5ml.

9) Indirect infectivity assay in primary monkey kidney cells by the

Interference Technique

7-10 day old primary Petas monkey kidney cultures in 6" x 2½" test tubes
were changed to maintenance medium (1.5ml/tube). See Table 1 (b).
Dilutions of virus were prepared in buffered 199 medium and 0.5 ml.
aliquots were inoculated into the tubes, a minimum of 4 tubes per dilution
being used and a further 6 tubes being retained as uninoculated controls.
The cultures were incubated on a roller apparatus (Appendix) at 36.5°C.
The cultures were examined for cytopathic effects of commonly occurring
monkey agents at 7 days post-inoculation and if clear were changed to
fresh medium. After a further 3 days the tubes were again changed to
100 TCD$_{50}$ of the virus. The challenge virus was also titrated at this time in cultures of the same batch which had been retained for this purpose, 0.5 ml. volumes of the virus diluted in buffered 199 medium being inoculated into tubes containing 1.5 ml. of medium. Final readings were made at 14-16 days post inoculation and titres were calculated by the method of Karber (1931) and expressed as IND$_{50}$/0.5ml. M6 titres were expressed as TCD$_{50}$/0.5ml.

10) Treatment of sera for use in overlay medium

Acid-washed Kaolin was brought to pH7 by repeated washing in phosphate buffered saline and was prepared as a 25% suspension in the same buffer. The suspension was sterilized by autoclaving and added to the serum samples in a 3 to 1 ratio. The Kaolin-serum mixture was shaken vigorously and incubated at room temperature for 20 minutes. The Kaolin was removed by centrifugation at 2000 rpm for 15 minutes on an M.S.E. Major centrifuge. The supernatant thus produced was a one in four dilution of the original serum, and the volumes added to the overlay media were adjusted accordingly.
Results

1) Investigation of factors influencing plaque formation

A) Source of GL-RK\(_{13}\) cells

A mycoplasma-free line of GL-RK\(_{13}\) (NIH) cells was compared to the laboratory line of GL-RK\(_{13}\) (CTR) in a rubella plaque test. No differences were observed in plaque morphology or plaque count over a series of five tests. The GL-RK\(_{13}\) (NIH) cells were selected in preference to the GL-RK\(_{13}\) (CTR) cells known to be contaminated with M. orale. GL-RK\(_{13}\) (MRC) also contaminated with M. orale and M. hominis were compared to the GL-RK\(_{13}\) (NIH) line in the plaque test. It was found that the plaques produced in this line were smaller than those seen in the GL-RK\(_{13}\) (NIH) cells, but the size differences noted between strains of rubella virus were still apparent. Figure 1.
Figure 1. Plaques produced by HPV-77 and RA27/3 strains in the NIH and MRC lines of GL-RK13 cells; (A) HPV77 in NIH cells (B) HPV77 in MRC cells (C) RA27/3 in NIH cells (D) RA27/3 in MRC cells.
Growth conditions for stock cultures and monolayers for infection

i) Serum incorporated in the medium

Stock cultures of GL-RK₁₃ (NIH) cells were grown on Eagles MEM supplemented with the uninaected sera; adult bovine, foetal or agamma calf serum. The average plaque size and count was determined using 3 strains of rubella virus. Four plates grown on each serum were inoculated with the standard control viruses SI (RA27/3) or AR1 (HPV-77) and 6 plates were inoculated with the Dunning strain. The optimum size and clarity of plaques was obtained in cultures grown on medium containing adult bovine serum, the results for foetal and agamma serum being identical. Table 4 shows the results obtained in plates grown on uninaected adult bovine and foetal calf serum, whilst Figure 2 shows the plaque morphology seen in these two systems. Optimum results were seen in cultures grown on uninaected adult bovine serum, therefore this serum was used in all further growth medium for GL-RK₁₃ (NIH) cells.
Figure 2. Standard control virus AR1 (HPV-77). Plaques produced in monolayers of GL-RK₁₃ cells grown on a) Uninactivated adult bovine serum, b) Uninactivated foetal calf serum.
<table>
<thead>
<tr>
<th>STRAIN OF VIRUS</th>
<th>ADULT BOVINE SERUM</th>
<th>FETAL Calf SERUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVERAGE PLAQUE NO.</td>
<td>AVERAGE PLAQUE SIZE</td>
</tr>
<tr>
<td>HPV-77 (AR1)</td>
<td>52.7</td>
<td>2.00-2.25mm</td>
</tr>
<tr>
<td>RA27/3 (S1)</td>
<td>22.0</td>
<td>1.25mm</td>
</tr>
<tr>
<td>DUNNING AT P4 in RK13</td>
<td>35.6</td>
<td>2.25-2.50mm</td>
</tr>
</tbody>
</table>

TABLE 4  Variation in plaque size and count obtained in cultures grown in the presence of varied sera.
ii) Cell seeding rate

The optimum seeding rate was determined in a comparative study using 35 mm dishes. Cell concentrations in excess of $1.5\times10^6$ cells in 8 ml. of medium produced uneven monolayers, showing particularly heavy growth in the centre of the plate. This thickened area either degenerated during maintenance or adhered closely to the overlay and was damaged during its removal prior to staining. Cell sheets of uneven density stained poorly and produced considerable variation in plaque morphology. Plates receiving less than $0.5\times10^6$ cells either did not reach confluency before showing degenerative changes or required longer than 3-5 days to become confluent and often did not survive the subsequent maintenance period. A seeding rate of $1\times10^6$ cells was selected as giving an even monolayer, confluent in 2-3 days. A similar quality of sheet could be obtained in 60mm plates seeded with $1.5\times10^6$ cells. The volume of cell suspension used to seed the plates was also of importance; volumes in excess of 4 to 5 ml. in a 35mm plate and 9-10 ml. in a 60mm plate often resulted in uneven monolayers due to aggregation of cells prior to settling and also uneven settling due to convection currents and wave motions within the greater depth of fluid, possibly set up by vibrations in the incubator. The optimum quality of cell sheet was obtained using volumes of 3 or 4 ml. for a 35mm dish and 8 ml. for a 60mm dish. Great care had to be exercised to ensure that the cell suspension was evenly dispensed before settling out as the cells showed a tendency to accumulate in the centre of the plate due to the circular motion in the fluid when the plates were moved. Gentle rocking of the plates as they were loaded into the incubator helped to overcome this problem. In order to demonstrate clear uniform plaques of any strain of rubella virus, a completely confluent even monolayer of GL-RK_{13} cells was necessary.
iii) Incubation conditions

It was necessary to avoid direct transfer of heat from the copper trays of the incubator, which was thought to damage Vero cultures in Falcon plastic (Ross, personal communication 1968), thus all dishes were laid on 0.25" thick perspex trays before being placed in the incubator. It was noted that Falcon plastic could be warped when placed directly on metal shelves and thus could result in uneven settling of cells. The 18" square perspex trays were drilled through at 2" intervals with ¼" holes to allow free circulation of the atmosphere within the cabinet. Where it was necessary to stack trays, supports were included which allowed at least 1" clearance between upper and lower trays, otherwise variations in the pH of the medium occurred due to poor gas circulation. A second pump for circulating the atmosphere within the incubator was included in order to prevent layering of the \( \text{CO}_2 \) gas in a full cabinet. Figure 3 shows the circulation and pH monitoring set up in the modified L.T.E. incubator. The set up was such that the flow of \( \text{CO}_2 \) gas to the cabinet was controlled by a pH meter which turned on the \( \text{CO}_2 \) flow as the pH rose and switched off as it fell back to 7.2. The system could be used without the pH meter control with some success simply by maintaining a very slow flow of gas to the cabinet and flushing with more \( \text{CO}_2 \) if the cabinet was opened.
Figure 3. CO$_2$ circulation and pH monitoring system in L.T.E. incubator.

a: air filters
v: gas valve, operated by pH meter
f: gas flow indicator bottle
w: water trap
p: pump
i: pH indicator medium and pH probe
m: pH meter and gas flow control
t: water tray for humidification
C) **Conditions for infection and maintenance of monolayers**

i) **Washing of monolayers**

It was noted that the plaques seen in cultures which had not been washed prior to infection were sometimes smaller and less distinct than those seen in washed monolayers, also plaque counts were sometimes appreciably lower, although this may have been a reflection of the difficulty of counting indistinct plaques. It was decided that all monolayers must be washed at least once prior to infection, firstly to remove accumulated cellular debris which could damage or obscure the cell sheet when stained and secondly to remove the adult bovine serum used for growth of the monolayers for it was occasionally found that this serum contained non-specific inhibitors of rubella virus.

(See paragraph iv (a)).

ii) **Volume of inoculum**

A dose-response curve was constructed for 4 volumes of inoculum for the SI (RA27/3) and AR1 (HPV-77) Standard Control viruses, Figure 4. Inoculum volumes greater than 0.1 ml/35mm plate gave plaque counts lower than the expected number. An inoculum volume of 0.1 ml was adopted for all further work.

iii) **Adsorption time**

Thirty six plates of GL-RK\textsubscript{13} were each inoculated with a constant volume of one dilution of the standard virus SI (RA27/3). Groups of 6 plates were overlaid after stated periods of adsorption from 10 to 60 minutes as room temperature. Figure 5 shows the average plaque counts obtained between 10 and 60 minutes. Maximum plaque counts were reached after 20 minutes adsorption.
Figure 4. Theoretical and actual plaque counts from 4 volumes of inoculum of S1 (RA27/3) standard virus and AR1 (HPV-77) standard virus in GL-RK cells.

- Theoretical plaque count for S1 virus
- Actual plaque count for S1 virus
- Theoretical plaque count for AR1 virus
- Actual plaque count for AR1 virus
Figure 5. Relationship of adsorption time to average number of plaques appearing per plate.
iv) Overlay Medium

a) Serum

Foetal and agamma calf serum, adult bovine, rabbit and goat were used, both before and after treatment with Kaolin powder, in the overlay medium. 3 x 60mm plates each inoculated with 0.2ml. of the same dilution of the standard virus ARL (HPV-77) were included in each serum group. Use of adult bovine, rabbit or goat serum in the overlay resulted in a reduction in the size and quality of the plaques.

Table 5. Prior treatment of these sera with Kaolin, improved the results but did not give plaques as satisfactory as those obtained with agamma calf serum. It should be noted that the use of diluted Kaolin-treated serum necessitated reduction in the distilled water used to make up the overlay medium, and as the serum was diluted with phosphate buffered saline, the toxicity of the medium was probably altered and the phosphate content increased; this may account for the fact that Kaolin treatment did not result in sera giving optimum results. Overlays containing foetal calf serum resulted in plaques of similar quality to those produced under medium containing agamma calf serum.

<table>
<thead>
<tr>
<th>SERUM IN OVERLAY</th>
<th>UNTREATED SERUM</th>
<th>KAOLIN TREATED SERUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLAQUE SIZE</td>
<td>PLAQUE COUNT</td>
</tr>
<tr>
<td>Agamma Calf</td>
<td>2.00-2.25mm</td>
<td>83</td>
</tr>
<tr>
<td>Adult Bovine</td>
<td>1.25-1.50mm</td>
<td>57</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.50-1.00mm</td>
<td>ND *</td>
</tr>
<tr>
<td>Goat</td>
<td>0.50-1.00mm</td>
<td>ND</td>
</tr>
</tbody>
</table>

TABLE 5   The plaque size and average count of ARL (HPV-77) virus obtained under overlays containing varied sera.

* Not done
b) Sodium Bicarbonate

Two concentrations of bicarbonate were compared, 0.044% and 0.088%; standard dilutions of the two control viruses SI (RA27/3) and AR1 (HPV-77) were inoculated onto each of 4 plates per bicarbonate sample. Size estimates quoted are the average from 20-30 plaques.

A lower concentration of NaHCO₃ in the overlay resulted in a reduction and variation in the size and clarity of the plaques. Table 6. Once the definition of the plaques became reduced and variable it was difficult to make an accurate count and useful assessment of plaque size.

<table>
<thead>
<tr>
<th>STRAIN OF RUBELLA</th>
<th>PLAQUE SIZE UNDER OVERLAYS CONTAINING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.044% NaHCO₃</td>
</tr>
<tr>
<td></td>
<td>0.088% NaHCO₃</td>
</tr>
<tr>
<td>SI (RA27/3)</td>
<td>0.75 - 1.50mm</td>
</tr>
<tr>
<td></td>
<td>1.00 - 1.25mm</td>
</tr>
<tr>
<td>AR1(HPV-77)</td>
<td>1.00 - 2.00mm</td>
</tr>
<tr>
<td></td>
<td>2.00 - 2.25mm</td>
</tr>
</tbody>
</table>

TABLE 6. The effect of sodium bicarbonate concentration in the overlay on the plaque size of SI (RA27/3) and AR1 (HPV-77) viruses.
Nearly half of the batches of Difco Noble Agar encountered in this study were found to contain some factor which inhibited optimum plaque formation. Plaques of variable and reduced size and count were obtained for SI (RA27/3) and AR1 (HPV-77) viruses, thus all batches of agar were screened against these in a plaque test before being accepted for routine use.

The Difco Noble Agar powder used throughout was made up at 3% (weight/volume) concentration in distilled water and stored in 40 ml. volumes at 4°C. Agar was melted immediately prior to use and great care was taken that the heating period should not be longer than necessary and normally did not exceed 15 minutes. Molten agar was maintained in a 60°C water bath until incorporated in the overlay medium for immediate use. Unused agar was discarded.

The volume and hence the depth of overlay used, had a marked effect on plaque size. Table 7. Volumes of 15 ml., or less than 6 ml. in a 60mm dish showed a reduction and variation in plaque size and morphology, furthermore the cell sheets were less uniform and tended to deteriorate during maintenance.

<table>
<thead>
<tr>
<th>VOLUME OF OVERLAY</th>
<th>SIZE OF HPV-77 PLAQUES</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 ml</td>
<td>1.50 - 2.00 mm*</td>
</tr>
<tr>
<td>10 ml</td>
<td>2.00 - 2.25 mm</td>
</tr>
<tr>
<td>8 ml</td>
<td>2.00 - 2.25 mm</td>
</tr>
<tr>
<td>6 ml</td>
<td>1.75 - 2.25 mm</td>
</tr>
<tr>
<td>5 ml</td>
<td>1.50 - 2.00 mm*</td>
</tr>
<tr>
<td>4 ml</td>
<td>1.0 - 1.25 mm*</td>
</tr>
</tbody>
</table>

TABLE 7. Relationship of volume and depth of overlay to size of plaques. 4 x 60mm plates per sample, 30-40 plaques per plate.
* Plaques very indistinct, edges not well defined.
Thus an overlay volume of 6 ml was used for all further work with 60 mm plates, similar results were achieved in 35 mm plates with overlay volumes of 3 or 4 ml. The overlay was introduced onto the plates at a temperature of 40-50°C, because temperatures in excess of this could conceivably increase inactivation of virus not fully adsorbed or damage the cell sheet. Plates were not moved to the incubator until the overlay had gelled as movement of plates containing semi-solid medium could easily damage the cell sheet.

v) **Incubation conditions**

Incubation was carried out at 32.5°C as the clarity of S1 (RA27/3) plaques was optimum at this temperature, whilst the plaque size and counts of other strains did not differ between 32.5°C and 36.5°C. Monolayers of GI-RK13 were infected with a standard inoculum of S1 (RA27/3) and AR1 (HPV-77) viruses and plates inoculated with each strain were stained daily from 3 to 7 days post inoculation. Figure 6 shows the percentage of plaques present each day taking the count at day 7 as 100%. The optimum time of incubation was found to be within 6-8 days for S1 (RA27/3) and days 7 and 8 for AR1 (HPV-77). In general day 7 was selected as the optimum time of staining, for although the plaque size increased after this time the edges of the plaques began to break down making size estimation difficult, and small secondary plaques appeared by the 9th and 10th day. Maintenance of infected cultures beyond 10 days was impractical due to deterioration of the cell sheet.
Figure 6. Increase in plaque count with days post-infection.

PERCENTAGE OF PLAQUE COUNT AT DAY 7

- HPV-77
- RA27/3

DAYS POST INFECTION
Sensitivity of the modified test as a titration system

A) Dose-response relationship

Plate cultures were inoculated with increasing dilutions of S1 (RA27/3) and a vaccine pool of RA27/3 virus and the dose-response relationships obtained are shown in Figure 7. The results suggest that the plaque count is directly related to the relative virus concentrations and that each plaque is specific and formed from a single infectious unit.
Figure 7. Dose-response line for two pools of RA27/3 virus.
B) Specificity of plaques

Equal volumes of standard dilutions of S1 (RA27/3) and AR1 (HPV-77) and varied dilutions of 2 rubella antisera were mixed and held at 32.5°C for 60 minutes, at which time 0.1 ml. volumes of each mixture were inoculated onto 2 x 35 mm plates of GL-RK<sub>13</sub> cells. A similar mixture of each virus and pre-immune sera were treated in the same manner. Table 8 shows the average plaque count from each mixture.

<table>
<thead>
<tr>
<th>VIRUS/SERUM MIXTURE</th>
<th>AVERAGE PLAQUE COUNT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/4</td>
</tr>
<tr>
<td>RA27/3/R50/70 AS</td>
<td>0</td>
</tr>
<tr>
<td>RA27/3/Pre-immune</td>
<td>35</td>
</tr>
<tr>
<td>HPV-77/J27/3 AS</td>
<td>ND</td>
</tr>
<tr>
<td>HPV-77/Pre-immune</td>
<td>ND</td>
</tr>
</tbody>
</table>

TABLE 8. Reduction of plaque count with specific Rubella antiserum.

* Not done.

The reduction in average count indicates that the plaques were probably due to an infection with rubella virus.
C) Reproducibility of the Test

i) Variation of the plaque count

The values for the titre of S1 (RA27/3) and AR1 (HrV-77) were taken from 100 separate tests and the Standard Deviations calculated:

<table>
<thead>
<tr>
<th>STRAIN OF VIRUS</th>
<th>NO. OF OBSERVATIONS</th>
<th>STANDARD DEVIATION</th>
<th>% + 1xS.D.</th>
<th>% + 2xS.D.</th>
<th>% + 3xS.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1(RA27/3)</td>
<td>100</td>
<td>3.51 ± 0.148</td>
<td>69%</td>
<td>93%</td>
<td>7%</td>
</tr>
<tr>
<td>AR1(HPV-77)</td>
<td>100</td>
<td>3.55 ± 0.176</td>
<td>75%</td>
<td>100%</td>
<td>-</td>
</tr>
</tbody>
</table>

TABLE 9. Standard deviations for titration of standard pools of RA27/3 and HPV-77 strains. Titres obtained as Log_{10} pfu/0.5ml in 100 separate tests carried out on GL-RK_{13} (NIH) cells.

The results of such an assay system are considered significant if 33% of all observations lie within one standard deviation of the mean, 66% within two standard deviations and 99% within 3 standard deviations from the mean, thus the titration system under study lies within these limits. In this investigation each plaque test included an assay of the standard pools of virus; tests showing titres for these pools which lay more than 3 Standard Deviations from the mean value quoted above were considered invalid.
Frequency distribution of plaque counts within one test and between consecutive tests.

Histograms of the distribution of plaque counts obtained from a single dilution of AR1 (HPV-77) and SI (RA27/3) within one plaque test and consecutive tests performed at weekly intervals are shown in Figures 8, 9 and 10. Figure 8 shows the distribution obtained within one test from the count of 48 plates each receiving 0.1 ml of one dilution of AR1 (HPV-77) virus. Figure 9 shows the distribution of plaque counts from 24 plates each receiving 0.1 ml of one dilution of SI (RA27/3) virus, within one test, whilst Figure 10 gives the distribution of SI (RA27/3) plaque counts from forty-eight consecutive tests containing 3 plates, each of which received 0.1 ml of one dilution of virus. The histograms obtained show an acceptable distribution.
Figure 8. Frequency distribution of plaque counts within one test. AR1 (HPV-77) Standard virus.
Figure 9. Frequency distribution of plaque counts within one test
S1 (RA27/3) Standard virus.
Figure 10. Frequency distribution of plaque counts between 48 consecutive tests. S1 (RA27/3) Standard virus.
D) Comparison with alternative methods of assay.

Table 10 shows the titres obtained for AR1 (HPV-77) virus in the modified GL-RK\textsubscript{13} plaque test, the GL-RK\textsubscript{13} microtitre system and the Patas Interference titration system carried out on 4 separate occasions. The results for infectivity assay by the plaque test compare favourably with the alternative methods available.

<table>
<thead>
<tr>
<th>TITRES OBTAINED FOR AR1 STANDARD VIRUS BY:</th>
<th>GL-RK\textsubscript{13} PLAQUES</th>
<th>GL-RK\textsubscript{13} MICROTI TRE</th>
<th>PATAS INTERFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.52 pfu/0.5ml</td>
<td>3.57 TC\textsubscript{50}/0.5ml</td>
<td>4.00 IND\textsubscript{50}/0.5ml</td>
<td></td>
</tr>
<tr>
<td>3.50 pfu/0.5ml</td>
<td>3.32 TC\textsubscript{50}/0.5ml</td>
<td>3.00 IND\textsubscript{50}/0.5ml</td>
<td></td>
</tr>
<tr>
<td>3.82 pfu/0.5ml</td>
<td>3.57 TC\textsubscript{50}/0.5ml</td>
<td>3.50 IND\textsubscript{50}/0.5ml</td>
<td></td>
</tr>
<tr>
<td>3.72 pfu/0.5ml</td>
<td>3.70 TC\textsubscript{50}/0.5ml</td>
<td>3.50 IND\textsubscript{50}/0.5ml</td>
<td></td>
</tr>
<tr>
<td>Mean:3.63 pfu/0.5ml</td>
<td>Mean:3.54 TC\textsubscript{50}/0.5ml</td>
<td>Mean:3.50 IND\textsubscript{50}/0.5ml</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 10 Titres of standard control virus pool, as determined in 3 titration systems.
3) Methods of measurement of the diameter of plaques and the reproducibility of the values obtained

The clarity of plaque formed by any one strain, at a particular passage level, although constant within one test, varied in consecutive tests, as the number of cells surviving within the perimeter of the plaque varied. Occasionally small foci of darker staining cells were seen within the plaque, Figure 11. With these results in mind, it did not seem feasible to employ the more sophisticated methods of plaque size measurement based on the assay of dye uptake by surviving cells. (Finter, 1969). Usually measurement was simply carried out with the aid of a transparent ruler, the plate being supported on a glass topped box, evenly illuminated from below by a fluorescent tube light source. The accuracy of the technique was not sufficient for finer measurement, but provided a reasonable estimate of the size range.

A projection technique of measurement was attempted with the aid of a simple photographic slide projector, however the magnification involved in projecting an image of the cell sheet onto a screen was so great that at high magnification it was difficult to distinguish the edges of a plaque and measurement became more difficult, see Figure 12.

More recently a Zeiss-Jena projection microscope became available allowing projection with magnification from x 17.5 to x 6.5. Measurements were repeated at the x6.5 magnification and the range of plaque sizes quoted for the individual strains were confirmed. However, even at as low a magnification as x6.5 the edges of the plaque were again difficult to locate exactly and estimations were taken conservatively resulting in marginally smaller values for the diameter of the plaques examined.

Figures 13 to 18 show the distribution of plaque sizes for 6 strains of rubella virus, measured on the projection microscope at this magnification within one test and between consecutive experiments. The histograms obtained show an acceptable size distribution.
Figure 11. Standard control virus AR1 (HPV-77). Foci of darker staining cells within the perimeter of the plaques.
Figure 12. Standard control virus AR1 (HPV-77). Plaques produced in GL-RK$_{13}$ cells, magnified x 10.
Figure 13. Frequency distribution of plaque size of AR1 (HPV-77) Standard virus; (A) within one test, 22 plaques measured on one plate, (B) within consecutive tests, 230 plaques measured on 19 plates, each from a separate test.
Figure 14. Frequency distribution of plaque size of S1 (RA27/3) Standard virus; (A) within one test, 28 plaques measured on one plate, (B) within consecutive tests, 108 plaques measured on 10 plates, each from a separate test.
Figure 15. Frequency distribution of plaque size of Cendehill virus; (A) within one test, 15 plaques measured on one plate, (B) within consecutive tests, 38 plaques measured on 4 plates, each from a separate test.
Figure 16. Frequency distribution of plaque size of Sheppard virus, (A) within one test, 18 plaques measured on one plate, (B) within consecutive tests, 86 plaques measured on 11 plates, each from a separate test.
Figure 17. Frequency distribution of plaque size of Dinning virus; (A) within one test, 16 plaques measured on one plate, (B) within consecutive tests, 44 plaques measured on 4 plates, each from a separate test.
Figure 18. Frequency distribution of plaque sizes of Janine virus; (A) within one test, 10 plaques measured on one plate, (B) within consecutive tests, 24 plaques measured on 4 plates, each from a separate test.
Discussion:

Taylor-Robinson et al (1964) suggested that the strain of GL-RK_{13} used might well influence plaque formation and in this study it was possible to demonstrate plaque formation in 3 strains of GL-RK_{13}, 2 of which were contaminated with mycoplasma. The original strain received from Taylor-Robinson, GL-RK_{13} (CTR) showed plaques indistinguishable from those seen in the GL-RK_{13} (NIH) line received from the U.S.A., only the GL-RK_{13} (MRC) line, originally propagated at R.I.T. in Belgium showed plaques which were smaller and clearer i.e. with fewer cells surviving within the perimeter. The difference in plaque size between strains of rubella virus was seen in all the strains of GL-RK_{13} cells examined suggesting that these differences were not peculiar to one cell strain.

Taylor-Robinson (Personal communication 1969) has proposed that the nature of the cytopathic effect of rubella virus in GL-RK_{13} cells requires a high population pressure of cells around the infected focus. In time lapse filming (McCarthy, 1969) cells are seen to move into the infected area, piling into the characteristic foci which are seen in direct visualization or are removed with the agar overlay in the plaque test, thus the denser the cell population around the focus, the higher will be the number of cells moving into the infected area and theoretically, the cytopathic effect should become more distinct. This idea would seem to be supported by the observations noted here with the plaque test, where optimum clarity of plaques was achieved with a completely confluent dense monolayer. It has been reported that too dense a monolayer can inhibit plaque formation (Taylor-Robinson et al, 1964) but this phenomenon was not seen with the cell concentrations used in this investigation.

Over-seeded plates maintained poorly and were usually damaged, however it was noted that there was a sharp increase in non-specific aggregation and clumping of GL-RK_{13} cells in an over-seeded culture maintained under
fluid overlay, which could easily mask true cytopathic effects and such a phenomenon may occur in the plaque test at higher cell seeding rates.

Sellers and Stewart (1959) reported that washing of pig kidney cell monolayers prior to infection with foot and mouth disease virus could result in a two fold increase in the plaque count, however Cooper (1957) had shown that such a procedure could even decrease the plaque count of vesicular stomatitis virus in chick embryo fibroblasts. Valle (1971) was able to demonstrate a relationship between the age of chick embryo fibroblast culture and the response to washing prior to infection, the older the monolayer the greater the increase in plaque count of vesicular stomatitis virus. It has been shown in this investigation that there is an enhancement of clarity of the rubella plaques and possibly of the count obtained in GL-RK_{15} cells which are washed prior to infection. It was thought that the effect was due to removal of cellular debris and inhibitors present in the serum used for growth of the monolayers. Unfortunately, the comparison of washed to unwashed cultures was not made after growth of the monolayers in medium containing serum treated to remove such non-specific inhibitors. Kato and Eggers (1969) reported the appearance of 2 factors in the medium from older chick embryo fibroblasts which could enhance interferon production and reduce susceptibility of the cells to virus infection. It is possible that such factors might influence plaque production in a cell system, thus washing of monolayers prior to infection may reduce these factors and yield higher plaque counts, the enhancing effect of washing being then related to the age of the cultures as reported by Valle (1971). There are no reports in the literature of similar findings in other cell systems but existence of such factors might well influence plaque production in continuous cell lines such as the GL-RK_{15}.

Valle (1971) found that the highest relative plaque counts were achieved in his system, with volumes of inoculum less than 0.05 ml, and
Similar results were found in this study. Valle (1971) postulated that the increased depth of fluid resulting from a larger volume of inoculum meant that some virus particles would take longer to reach the monolayer and in fact that a proportion of particles might never settle on the cell sheet. Certainly with adsorption times as short as twenty to thirty minutes, as used in this study, an increasing depth of fluid would mean a reduction in the number of particles reaching the cell sheet within the time allowed.

Adsorption times of up to 2 hours for rubella virus in GL-RK\textsubscript{13} cells have been reported (Hekker et al, 1969) in the literature, however, in this study it was found that maximum plaque numbers were reached within 10-20 minutes. Valle (1971) reported similar findings for vesicular stomatitis virus in chick embryo fibroblasts with the majority of virus being adsorbed within 10 minutes at 37°C. It is unlikely that 100% of either rubella virus or vesicular stomatitis virus is adsorbed within 10 minutes, for the rate of movement of virus through the fluid film on the monolayer would not be this rapid, however Cooper (1955) showed that the latter virus was able to migrate through an agar overlay and thus it was possible that adsorption and infection of the monolayer continued after overlay. It is possible that a similar phenomenon occurs with rubella virus in the GL-RK\textsubscript{13} plaque test for diffusion of the virus is suggested by the appearance of secondary plaques in monolayers maintained for longer than 9-10 days.

The composition of the maintenance overlay was found to be of importance. The optimum bicarbonate concentration was found to lie within narrow limits, it was thought that this was a function of maintenance of the cell sheet for on occasions a HEPES based medium with a greatly reduced bicarbonate concentration has been tried with equal success. Rabbit, goat and adult bovine serum were found to
was partially removed by prior kaolin treatment of the sera. Adult bovine serum gave optimum results when used in the growth medium for production of monolayers, which were washed prior to infection, suggesting that the inhibitory factor acted directly on some function of the virus and not the cell system.

Many papers appear in the literature concerning the presence of inhibitory factors in agar, (Takemoto and Liebhaber, 1961, 1962; Agol and Chumakova 1963, Vaheri, Sedwick and Plotkin, 1967) these are generally considered to be sulphated polysaccharides which inhibit the growth of certain viruses. Many additives and alternatives have been put forward (Valle, 1971) such as DEAE-dextran, and carboxymethyl cellulose as well as purified forms such as agarose. Similar inhibitory batches of agar were encountered in this investigation often associated with overheating of the agar during sterilization. Fogel et al (1969) reported an increase in plaque size and count for strains of rubella virus under agar overlays containing DEAE-dextran, this however was not investigated in this study, and it is not known whether such additions would have overcome the toxicity of some batches of agar or enhanced plaque formation under non-toxic batches.

In general the results show that the modified GL-RK plaque test for titration of rubella virus is sensitive, specific and statistically reliable, and furthermore, that the quantitative values obtained by this technique compare favourably with those resulting from existing assay methods. The measurement of plaque diameters with the naked eye was confirmed by projection and measurement of the magnified plaques. Distribution histograms of the plaque sizes measured for 5 strains of rubella virus show a good peak in the region of diameters quoted and suggest that the plaque size differences noted are real and not the result of variable morphology in an unreliable test system.
Thus modification of the original plaque test technique has resulted in an improved system giving reliable and reproducible results for both plaque morphology and assay of the virus.
SECTION II

The Cytopathic effects of rubella virus strains in selected tissues.
Introduction

Materials and Methods

1) Plaque test for rubella virus in
   A) GL-RK_{13} cells
   B) Vero cells

2) Preparation and infection of cultures of GL-RK_{13} cells for the comparison of cytopathic effects.

3) Preparation and infection of cultures for the passage of strains of rubella virus,
   A) GL-RK_{13}
   B) Primary Erythrocebus patas monkey kidney
   C) Vero
   D) BHK21/13S
   E) WI-38

Results

1) Plaque morphology of low pass strains in GL-RK_{13} cells

2) Plaque morphology of laboratory adapted strains in GL-RK_{13} cells

3) Plaque morphology in GL-RK_{13} cells of selected strains after passage in various culture systems

4) Morphology of the microfoci produced by selected strains of rubella in GL-RK_{13} cells maintained under fluid overlay

5) Plaque morphology of selected strains of rubella in Vero cells.

Discussion
Rubella virus is known to replicate in a wide variety of cell and tissue cultures, but in a very few is a distinct cytopathic effect seen, (McCarthy & Taylor-Robinson, 1967); hence many laboratories choose to remain dependent on an indirect assay system based on the original interference technique of Parkman et al (1962). Cytopathic effects are seen in certain primary tissue cultures such as human amnion (Weller et al, 1962), adult human thyroid, (McCarthy et al, 1963) rabbit kidney (Bellocourt, Wong & Walcroft, 1965) and rabbit embryonic cells (Reddick & Loesel, 1966), however the effects are usually slight and slow to appear and do not compare favourably with cell lines for ease of technical manipulation. Cell lines reported in the literature as subject to the cytopathic effects of rubella virus include those of monkey origin, LLC-MK2, GMK-BSC-1, (Veronelli & Maasab, 1965) GMK-AH-1, (Gunalp 1965) GL-V3A, (Beale & Christofinis, 1964) and Vero, (Lieshaber et al, 1969); those of hamster origin, BHK21, (Vaheri et al, 1965) and those of rabbit origin, GL-RK13 (McCarthy & Taylor-Robinson, 1965), GL-RK3 (Beale et al, 1964), and SIRC, (Leerhoy, 1965). Cell lines of monkey origin again demonstrate a slowness in the appearance of the cytopathic effect whilst the hamster line BHK/21 has proved difficult to handle reproducibly in inexperienced hands (Vaheri, personal communication, 1970). The SIRC line originating from rabbit cornea is again unreliable in laboratories inexperienced in its use (Leerhoy, personal communication, 1970) but does show a rapid progress of cytopathic effect leading to total degeneration. The GL-RK13 and GL-RK3 cell lines differ from the others described in that the rapidly progressing cytopathic effect would seem to be the result of an active accumulation of dead cells at the focus of infection rather than a simple destruction of the cell sheet resulting in a 'holed' appearance (McCarthy, 1969).
rubella virus have only been undertaken in BHK 21 cells (Plotkin, 1969) and GL-RK\textsubscript{13} cells (Butler & Laurence, 1966; Hekker et al, 1969; Oxford, 1969; Morgan, 1969; Fogel et al, 1969). Parkman et al (1964 first suggested that plaque formation and type might present a suitable marker of attenuation, they deduced this from their studies on the HPV vaccine strain which developed the capacity to form plaques in monolayers of GL-RK\textsubscript{13} cells after approximately 70 passages in primary monkey kidney tissue, whereas originally at pass two in this tissue, plaques were not detected in GL-RK\textsubscript{13}. Other possible indications of loss of virulence appeared to correlate with the acquired ability to produce plaques, (Parkman et al 1966 & 1969). Hekker et al, (1969) were able to confirm these results but found that the low pass virus did produce small indistinct plaques in GL-RK\textsubscript{13} cultures. Oxford (1969) reported that strains of rubella virus at low passage levels i.e. below pass 7 in tissue culture, produced little or no cytopathic effect in GL-RK\textsubscript{13} cells, but acquired this ability on passage in these cells. Butler et al (1966) suggested that the type of tissue selected for passage of a strain of rubella could influence the plaque type obtained in GL-RK\textsubscript{13} cultures under agar overlay, and whilst Morgan (1969) was able to demonstrate an increase in the cytopathic effect as measured by an increase in the size of plaque achieved after passage of one strain in GL-RK\textsubscript{13} tissue, he was unable to relate the differences in plaque morphology between other strains to their previous history.

Fogel et al (1969) encountered differences in the plaque type of the strains they investigated, when the solidifying agent for the overlay medium was agar or agarose, but found no variation in plaque morphology under carboxymethyl cellulose overlays suggesting that the differences encountered were a reflection of varying sensitivity of the strains to inhibitors present in the agar.
RA27/3 strain to produce plaques in BHK/21 cells, whereas the original strain below the eighth pass in WI-38 did not, similarly the other vaccine strains examined and 5 unattenuated strains of rubella virus did not plaque in BHK/21 cells, again suggesting a relationship between attenuation and the cytopathic effect produced in certain cell cultures.

It was not clear from the reports described above whether an increase in the size of plaques or the acquisition of the capacity to produce plaques in GL-RK or BHK 21 cultures was related to attenuation of rubella strains as a whole or whether it was a peculiarity of the individual strains examined or the cells selected. Neither was the influence of passage certain, either from the point of view of the tissue selected or the passage levels examined. No reports had appeared of a comparison of low pass rubella virus strains from varied geographical or clinical sources and similarly comparisons of strains under controlled passage had apparently not been attempted. It was not always clear exactly what the passage history of a strain was or at what point it was no longer considered low pass virus. Some of the work reported had only compared minimal and extensive cytopathic effect under fluid medium and possibly intermediate stages had not been distinguished as clearly as was possible on the grounds of plaque size. It appeared that the differences seen in plaque size under agar may only have been functions of the sensitivity of the strains to the agar overlay, therefore it would be interesting to see if the same differences were seen in other cell systems under the same overlay or under fluid medium. A survey of the cytopathic effects of strains of rubella from varied sources was undertaken together with a limited investigation into the influence of passage on these properties.
Materials and Methods: -

1) Plaque test for rubella virus in
   A) GL-RK\textsubscript{13} cells.
   The modified plaque technique is described in detail in
   Section I.

B) Vero cells.
   The test was based on the method of Rhim and Schell, (1967)
   and was carried out in 35mm Falcon plastic petri dishes, seeded with
   1 \times 10^6 cells contained in 4 ml. of growth medium. The plates were
   incubated in the modified LBE cabinet at a temperature of 32.5°C and
   pH of 7.2-7.4. The cultures being confluent in 72 hours were drained
   of growth medium, and washed once with buffered Eagles MEM before
   inoculation. Virus dilutions were made in buffered Eagles MEM and
   inoculated onto the centre of the plate in volumes of 0.1 ml.
   Adsorption was allowed for 30 minutes at room temperature before the
   plates were overlaid with 3 ml. of medium containing indicator-free
   Eagles MEM, 0.089\% NaHCO\textsubscript{3}, 2\% foetal or agamma calf serum and 1.5\%
   Difco Noble Agar. The plates were left at room temperature for a
   further 20 minutes to allow the overlay to solidify before being returned
   to incubation for 8 days at which time a second 2 ml. volume of overlay
   was introduced, containing neutral red at a concentration of 1 in 20,000.
   Incubation was continued for a further 3-4 days, at which time the
   plaques were clearly visible. Control cultures infected with HPV-77
   (AR1) and RA27/3 (S1) were included in every test.
2) Preparation and Infection of cultures of GL-RK_{13} cells for comparison of cytopathic effects.

6 x 8 test tubes were seeded with sufficient cells (2-4x10^5 cells) to become confluent in 48-72 hours, and incubated at 32.5°C in the stationary position. When confluent the cultures were drained, washed once with buffered Eagles MEM and refed with 2 ml. of the maintenance medium.

Each of 4 tubes received 0.2 ml. of a standard titre (3.0-3.5pfu/0.5ml) of one of the rubella virus strains under study and all of the strains investigated were always compared in one experiment. The tubes were returned to stationary incubation for a further five days at which time the cytopathic effect was evident but not extensive and secondary foci were not present. The tubes were drained and fixed in formalised phosphate buffered saline, after which the cytopathic effects could be compared and photographed.
3) Preparation and infection of cultures for the passage of strains of rubella virus

A) GL-RK

GL-RK cells were prepared as described in Section 2. Between two and four tube cultures were each inoculated with 0.2 ml. of the strain under study and returned to rolled incubation. When the cytopathic effect was extensive, usually 7-10 days, the fluids were harvested, pooled and dispensed into small volumes for storage at -70°C. Samples were immediately subjected to further passage as described above or stored until tissue was available, similarly samples were included in a plaque test or stored until they could be examined in this manner. All the strains examined were passaged at each level in one batch of tissue.

B) Primary Erythrocebus patas monkey kidney

Confluent 7 to 10 days old cultures in 6 x 5 tubes were drained of growth medium, washed once with buffered 199 medium, and refed with 1.8 ml. of maintenance medium.

The cultures were inoculated with 0.2 ml. volumes of virus and incubated as described in Section 3 (A). The cultures were harvested ten to fourteen days after infection, pooled and dispensed for storage at -70°C.

C) Vero

Tube cultures were prepared, infected and harvested by the methods described in Section 3, (A) above.
D) BHK-21/13S

The cultures were prepared, infected and harvested by the methods described in Section 3,(A)

E) WI-38

6 x 6 test tubes were seeded with $1 \times 10^5$ cells in order to become confluent in 3 to 4 days, and incubated in the stationary position. Confluent cultures were washed once with buffered Eagles MEM and refed with 2.0 ml. of maintenance medium. The cultures were infected and harvested by the methods described in Section 3,(A) above.
1) Plaque morphology of low-pass strains in GL-RK₁₃ cells.

The results obtained for the size of plaques produced by the low pass strains i.e. those with less than seven passages in any system, are shown in Table 11a and b, with details of the source and country of origin where this information was available.

All but 2 low pass strains from naturally acquired infections gave plaques in the 0.75-1.25mm range. Figure 19 a-f illustrate the typical plaques formed by a) Sheppard, b) Lesley, strains isolated from naturally acquired post-natal infection, c) Savva, and d) H.E. strains isolated from foetal tissue after intrauterine infection, and e) Thomas and f) Andrews, strains isolated from rubella syndrome infants. Only the Dunning strain isolated from a congenitally infected infant and the Janine strain from an infection contracted whilst handling, Dunning showed large clear plaques of 2.25-2.50 mm. Figure 20 a and b shows the plaques formed by these 2 strains. Strains isolated from experimentally infected monkeys and human vaccinees showed plaques typical of the strain administered in each case.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ORIGIN</th>
<th>COUNTRY &amp; YEAR OF ISOLATION</th>
<th>PLAQUE SIZE IN LM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isolated from natural infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAY GL-RK&lt;sub&gt;13&lt;/sub&gt; P&lt;sub&gt;3&lt;/sub&gt; *</td>
<td>Throat swab</td>
<td>U.K. 1967</td>
<td></td>
</tr>
<tr>
<td>SHEPPARD GL-RK&lt;sub&gt;13&lt;/sub&gt; P&lt;sub&gt;6&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JUDITH (A) YRK P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Lymph node</td>
<td>U.K. 1963</td>
<td>0.75 - 1.00</td>
</tr>
<tr>
<td>LESLEY C.S +</td>
<td></td>
<td>U.K. 1968</td>
<td></td>
</tr>
<tr>
<td>ROBB C.S.</td>
<td>Throat and nose swab</td>
<td>U.K. 1972</td>
<td></td>
</tr>
<tr>
<td>JANINE C.S.</td>
<td></td>
<td>U.K. 1968</td>
<td>2.25 - 2.50</td>
</tr>
<tr>
<td>BROWN MK P&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Throat swab</td>
<td>U.S.A. 1966</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Isolated from vaccinees</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.S. (CENDEHILL) GL-RK&lt;sub&gt;13&lt;/sub&gt; P&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>U.K. 1968</td>
<td>1.25 - 1.50</td>
</tr>
<tr>
<td>GEL (HPV-DUCK) GL-RK&lt;sub&gt;13&lt;/sub&gt; P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Throat swab</td>
<td></td>
<td>2.00 - 2.25</td>
</tr>
<tr>
<td>DOU (RA27/3) GL-RK&lt;sub&gt;13&lt;/sub&gt; P&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>U.K. 1970</td>
<td></td>
</tr>
<tr>
<td>GEDDES (RA27/3) C.S.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McNULTY (RA27/3 C.S.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Isolated from experimentally infected monkeys</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK6 (HPV-77) MK P&lt;sub&gt;1&lt;/sub&gt; **</td>
<td>Nose swab</td>
<td>U.K. 1968</td>
<td>2.00 - 2.25</td>
</tr>
<tr>
<td>MK 10 (SHEPPARD) MK P&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>U.K. 1968</td>
<td>0.75 - 1.00</td>
</tr>
</tbody>
</table>

* Passage level of strain when examined
+ Fresh clinical specimen examined
** Monkey kidney tissue.

Table 11 (a) Plaque size of low pass strains isolated from postnatally acquired infection.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ORIGIN</th>
<th>COUNTRY &amp; YEAR OF ISOLATION</th>
<th>PLAQUE SIZE IN MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated from Fetal tissues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HE GL-RK₁₃ P₁</td>
<td>Fetal kidney tissue</td>
<td>U.K.</td>
<td>1.00 - 1.25</td>
</tr>
<tr>
<td>WRIGHT GL-RK₁₃ P₁</td>
<td>Fetal liver tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAVVA C.S. +</td>
<td>Placenta</td>
<td>U.K. 1968</td>
<td>1.00</td>
</tr>
<tr>
<td>Isolated from rubella syndrome infants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LABBERT GL-RK₁₃ P₁</td>
<td>6 day infant</td>
<td>U.K. 1966</td>
<td></td>
</tr>
<tr>
<td>PULLEN GL-RK₁₃ P₂</td>
<td>Throat swab of 9 month infant</td>
<td></td>
<td>0.75 - 1.00</td>
</tr>
<tr>
<td>ANDREWS GL-RK₁₃ P₃</td>
<td>Throat swab of 6 day infant</td>
<td>U.K.</td>
<td></td>
</tr>
<tr>
<td>THOMAS (E) GL-RK₁₃ P₃</td>
<td>Lens tissue of 4 week infant</td>
<td>U.K.</td>
<td></td>
</tr>
<tr>
<td>DUNNING GL-RK₁₃ P₄</td>
<td>Throat swab of 6 month infant</td>
<td>U.K. 1967</td>
<td>2.25 - 2.50</td>
</tr>
<tr>
<td>THOMAS (T) GL-RK₁₃ P₇</td>
<td>Throat swab of 4 week infant</td>
<td>U.K.</td>
<td></td>
</tr>
<tr>
<td>SIMONI MK P₂ **</td>
<td></td>
<td>U.S.A. 1965</td>
<td></td>
</tr>
<tr>
<td>HITCHCOCK MK P₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GIGUERRE (1) MK P₂</td>
<td>Unknown</td>
<td>U.S.A. 1964</td>
<td>0.75 - 1.00</td>
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<tr>
<td>CARNWRIGHT MK P₂</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LEFEBVRE (1) MK P₃</td>
<td>4-6 week infant</td>
<td></td>
<td></td>
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<tr>
<td>GABRIEL MK P₃</td>
<td>Unknown</td>
<td>CANADA. 1965</td>
<td></td>
</tr>
<tr>
<td>GOLDTHORPE MK P₃</td>
<td>Throat swab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEFEBVRE (2) MK P₆</td>
<td>4-6 week infant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Passage level of strain when examined.
+ Fresh clinical specimen examined.
** Monkey kidney tissue

Table 11 (b) Plaque size of low pass strains isolated from intra-uterine infections.
Figure 19. Plaques produced by the low pass strains Sheppard (A), Lesley (B), Savva (C).

1cm
Figure 19. Plaques produced by the low pass strains H.E. (D), Thomas (E) and Andrews (F).

1 cm
Figure 20. Plaques produced by the Dunning strain (A) and the Janine strain (B).

1 cm
Plaque morphology of laboratory adapted strains in GL-RK\textsubscript{13} cells

The results obtained for the size of plaques produced by laboratory adapted strains i.e., those with more than 7 passages in any system, are shown in Table 12 with details of the origin and country of isolation. A wide range of plaque sizes were found with these strains.

Figure 21 shows the typical plaques produced by a) HPV-77, 2.0-2.25 mm, b) Cendehill, 1.25-1.50 mm, and c) RA27/3, 1.0-1.25 mm.

Figure 22 compares the plaques produced by the HPV-77 strain with those produced by this strain after a further 3 passes in primary rabbit kidney and by the HPV-150 strain after 12 further passes in chick embryo tissue culture, the plaques are apparently identical.

The Giguerre strain was unusual in that 2 types of plaque were seen, one of 1.0-1.25 mm. - the second of 2.00-2.25 mm, see Figure 23.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ORIGIN</th>
<th>COUNTRY &amp; YEAR OF ISOLATION</th>
<th>PLAQUE SIZE IN IU/50S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isolated from postnatal infection</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>JUDITH (B) GL-RK13 P50 *</td>
<td>Throat swab</td>
<td>U.K. 1963</td>
<td>2.50 - 3.00</td>
</tr>
<tr>
<td>CENDEHILL YRK+ P51</td>
<td>Throat swab</td>
<td>Belgium</td>
<td>1.25 - 1.50</td>
</tr>
<tr>
<td>HPV-77 MK** P78</td>
<td>Throat swab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-77 MK P78 + YRK P3</td>
<td>Throat swab</td>
<td>U.S.A. 1962</td>
<td>2.00 - 2.25</td>
</tr>
<tr>
<td>HPV-77 MK P150 + CETC ***P12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Isolated from intra uterine infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giguerre (2) MK P2 RK13 P7</td>
<td>Unknown</td>
<td>U.S.A. 1965</td>
<td>1.00 + 2.00 - 2.25</td>
</tr>
<tr>
<td>RA27/3 WI-38 P30</td>
<td>Foetal kidney</td>
<td>U.S.A.</td>
<td>1.00 - 1.25</td>
</tr>
</tbody>
</table>

* Passage history of strain when examined
+ Primary rabbit kidney tissue
** Monkey kidney tissue
*** Primary chick embryo tissue culture

Table 12. Plaque size of laboratory adapted strains.
Figure 21. Plaques produced by the HPV-77 strain (A), the RA27/3 strain (B) and the Cendehill strain (C).
Figure 22. Plaques produced by HPV-77 strain, grown in primary monkey kidney tissue (A), after a further three passes in primary rabbit kidney (B), and the HPV-150 strain after twelve passes in chick embryo tissue (C).
Figure 23. Plaques produced by the Giguere strain.
Plaque morphology in GL-RK, cells of selected strains after passage in various culture systems

Representative low pass and laboratory adapted strains were subjected to limited passage in primary Erythrocebus patas monkey kidney tissue, GL-RK, Vero, BHK21, and WI-38 cells. The plaque morphology of the strains were examined at each passage level.

Table 13 shows the plaque sizes recorded for each strain at the highest passage examined in each tissue. A change in plaque morphology was only seen in the Janine strain on passage in GL-RK cells, Figure 24; in the Dunning and Janine strains after passage in primary monkey kidney and Vero cultures, Figure 25 and 26; and in the Cendehill and Thomas strain after passage in BHK21 cells, Figures 27 and 28. These four strains were unchanged on passage in the other tissues selected and the remaining strains of rubella did not demonstrate an alteration in plaque morphology in any of the tissues examined.

The manner in which the change in plaque size occurred in GL-RK and primary monkey kidney tissue was of interest in that the same process appeared to happen for both Janine and Dunning strains. In these two strains the majority of plaques were large on the first pass, but small on the last passage levels tested, however at the intermediate levels both large and small plaques were present. Thus, for the Dunning strain in monkey kidney a small plaque appeared at the second passage level and rapidly became predominant so that by the fourth passage only small plaques were present. The changes seen on passage in BHK21 cells in the Thomas and Cendehill strains appeared to occur by a different process, such that only a small plaque was present at the first pass, but by the second passage the small plaques were present in approximately equal numbers with plaques in an intermediate size range, and by the third and
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>PLAQUE SIZE IN MM OF ORIGINAL MATERIAL</th>
<th>PRIMARY MK*</th>
<th>PASSAGE LEVEL &amp; PLAQUE SIZE (IN MM) IN:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Low pass strains isolated from postnatal infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHEPPARD</td>
<td>0.75 - 1.00</td>
<td>+P6, 0.75 - 1.00</td>
<td>P3, 0.75 - 1.00</td>
<td>P6, 0.75 - 1.00</td>
<td>P3, 0.75 - 1.00</td>
</tr>
<tr>
<td>LESLEY</td>
<td>0.75 - 1.00</td>
<td>P6, 0.75 - 1.00</td>
<td>P4, 0.75 - 1.00</td>
<td>P6, 0.75 - 1.00</td>
<td>P3, 0.75 - 1.00</td>
</tr>
<tr>
<td>JANINE</td>
<td>2.25 - 2.50</td>
<td>P6; 1.00 - 1.25</td>
<td>ND</td>
<td>P6, 1.00 - 1.25</td>
<td>ND</td>
</tr>
<tr>
<td>Low pass strains isolated from intra-uterine infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAVVA</td>
<td>1.00</td>
<td>P3, 1.00</td>
<td>P2, 1.00</td>
<td>P3, 1.00</td>
<td>ND</td>
</tr>
<tr>
<td>THOMAS (E)</td>
<td>0.75 - 1.00</td>
<td>P3, 0.75 - 1.00</td>
<td>P3, 0.75 - 1.00</td>
<td>P3, 1.75 - 2.50</td>
<td>P2, 0.75 - 1.00</td>
</tr>
<tr>
<td>DUNNING</td>
<td>2.25 - 2.50</td>
<td>P6, 1.00 - 1.25</td>
<td>P4; 1.00 - 1.25</td>
<td>P6, 2.25 - 2.50</td>
<td>P3, 2.25 - 2.50</td>
</tr>
<tr>
<td>Laboratory adapted strains</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFV-77</td>
<td>2.0 - 2.25</td>
<td>P6, 2.00 - 2.25</td>
<td>P5, 2.00 - 2.25</td>
<td>P6, 2.00 - 2.25</td>
<td>P3, 2.00 - 2.25</td>
</tr>
<tr>
<td>GENDENHILL</td>
<td>1.25 - 1.50</td>
<td>ND</td>
<td>P4; 1.25 - 1.50</td>
<td>P3, 1.25 - 1.50</td>
<td>P4, 2.50 - 3.00</td>
</tr>
<tr>
<td>RAC27/3</td>
<td>1.00 - 1.25</td>
<td>P6, 1.00 - 1.25</td>
<td>P3, 1.00 - 1.25</td>
<td>P6, 1.00 - 1.25</td>
<td>P3, 1.00 - 1.25</td>
</tr>
</tbody>
</table>

* Monkey kidney tissue
+ Passage level examined.

Table 13. Plaque size of selected strains of rubella after passage in various culture systems.
Figure 24. Plaques produced by the Janine strain after passage in GL-RK\textsubscript{13} cells.
Figure 25. Plaques produced by the Dunning strain after passage in primary monkey kidney tissue.
Figure 26. Plaques produced by the Janine strain after passage in primary monkey kidney tissue.
Figure 27. Plaques produced by the Cendehill strain after passage in BHK-21 cells.
Figure 28. Plaques produced by the Thomas strain after passage in BHK-21 cells.
fourth pass the majority were of the "new" larger plaque type with a few scattered small and intermediate size plaques. The two processes are most simply illustrated by a graph of the distribution of plaque sizes at each passage level. Figure 29 illustrates these results for Dunning virus passaged in primary Patas monkey kidney tissue culture and Cendehill passaged in BHK21 cells and the results for Dunning in GL-RK₁₃ are included in order to demonstrate the normal plaque size distribution. The numbers of plaques at each size were scored on 6 plates each of which had received 0.1 ml. inoculum of a strain at a stated passage level, there were approximately 20 plaques per plate.

In order to determine how stable the new plaque characteristics might be it was decided to passage the Dunning and Janine strains from the fifth pass in monkey kidney, back through GL-RK₁₃ again, testing the plaque morphology at each level. In both the Dunning and Janine strains after one GL-RK₁₃ pass the 1.00-1.25 mm. plaques had become 1.25-1.50 mm, by the second pass 1.50-1.75 mm, by the third pass 2.0-2.25 mm. Here there seemed simply to be a readaptation to the GL-RK₁₃ cell system with the growth in this tissue and hence the plaque size improving with each passage level.
Figure 29. Distribution of plaque sizes at each passage level for Dunning passed in GL-RK₁₃ and primary monkey kidney and Cendehill passed in BHK-21 cells.
4) The morphology of the microfoci produced by selected strains of rubella in GL-RK\textsubscript{13} cells maintained under fluid overlay.

The 3 vaccine strains of rubella, HPV-77, RA27/3 and Cendehill together with the low pass strains Dunning, Thomas and Lesley were inoculated into tube cultures of GL-RK\textsubscript{13} and the cytopathic effects examined and compared. The Dunning strain at the fourth pass in Vero cells and the Thomas strain at the third pass in BHK-21 cells were also included.

Those strains producing large plaques in GL-RK\textsubscript{13} cells, namely HPV-77, low pass Dunning and Thomas after passage in BHK-21 cells, showed a relatively wide spread of granular round cells around each foci of infection whilst those strains giving small plaques in GL-RK\textsubscript{13} cells, RA27/3, Lesley and Dunning after passage in Vero cells showed a more compact group of rounded cells with little scatter and spreading out from the focus. It was difficult to evaluate the microfoci produced by the Cendehill strain but it appeared to be closer to the compact focus produced by the small plaque types.

Figure 30 a-c illustrate the microfoci produced by the large plaquing strains HPV-77 and Thomas after passage in BHK-21 cells, whilst Figure 31 a-d illustrates the small and intermediate size plaque types, RA27/3, Lesley, Dunning after Vero passage, and Cendehill.
Figure 30. (A) Cytopathic effect of the HPV-77 strain in GL-RK₁₃ cells.
(B) Control uninoculated culture
Figure 30. (C) Cytopathic effect in GL-RK$_{13}$ cells, of the Thomas strain, after passage in BHK-21 cells.
Figure 31. Cytopathic effect in GL-RK13 cells of (A) The RA27/3 strain and (B) The Lesley strain.
Figure 31. Cytopathic effect in GL-RK\textsubscript{13} cells of (C) The Dunning strain after passage in Vero cells and (D) The Cendehill strain.
5) Plaque morphology of selected strains of rubella in Vero cells.

The strains examined in this system and the plaque size obtained are shown in Table 14 together with the plaque size obtained in GL-RK\textsubscript{13} cells.

<table>
<thead>
<tr>
<th>Strain of Rubella</th>
<th>Plaque Size in Vero Cells</th>
<th>Plaque Size in GL-RK\textsubscript{13} Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-77</td>
<td>1.25 - 1.5</td>
<td>2.0 - 2.25</td>
</tr>
<tr>
<td>RA27/3</td>
<td>1.25 - 1.5</td>
<td>1.0 - 1.25</td>
</tr>
<tr>
<td>CENDHILL</td>
<td>1.25 - 1.5</td>
<td>1.25 - 1.5</td>
</tr>
<tr>
<td>DUNNING (Pass 6 in RK\textsubscript{13})</td>
<td>1.25 - 1.5</td>
<td>2.25 - 2.50</td>
</tr>
<tr>
<td>LESLEY (Pass 1 in MK)</td>
<td>1.25 - 1.5</td>
<td>0.75 - 1.0</td>
</tr>
</tbody>
</table>

Table 14 Strains of rubella virus tested in the Vero plaque system, showing the size of plaques obtained in this test as compared to that seen in the GL-RK\textsubscript{13} system.

No variation in the plaque morphology of these strains could be found in the Vero cell system.
Some twenty-four low pass strains from naturally acquired cases of rubella infection were examined; all but 2 strains, Dunning and Janine, produced plaques in the 1.00 mm size range. These findings are in agreement with those of Oxford (1969) who reported that strains with fewer than 7 tissue culture passages produced little or no cytopathic effect in GL-RK<sub>13</sub> cells. No detectable difference could be found in the plaque morphology of low pass strains isolated from pre or post-natally acquired infections, neither could any variation be found between strains isolated from varied clinical sources, or from cases occurring in different years in the UK, USA or Canada. Furthermore the tissue selected for isolation of the strain, GL-RK<sub>13</sub> or monkey kidney, or the passage level at which they were examined, did not appear to influence the plaque morphology. The two low pass strains Dunning and Janine were unusual in that they gave large clear plaques of 2.25 - 2.50mm. It was possible that the Janine strain was in fact Dunning for the case of rubella from which it was isolated correlated neatly with a laboratory exposure to high titre samples of the Dunning strain. The unusual plaque character of these 2 strains in GL-RK<sub>13</sub> cells did not seem to be related to the tissue selected for isolation or the passage level of the original material examined, as similar strains isolated in the same laboratory in GL-RK<sub>13</sub> cells produced characteristic 1.00 mm plaques e.g. Day at pass 3, Sheppard at pass 6 and Pullen at pass 2.

It was possible that the large plaque type of the Dunning strain, isolated from a 6 month old rubella syndrome baby, was a result of the prolonged replication in utero or in the infant postnatally, however the Pullen strain isolated in the same laboratory was obtained from a 9 month old congenitally infected child and again produced
characteristic 1.00 mm. plaques. The possibility remains that some undetected factor or condition in the Dunning child had influenced the strain in some manner.

The strains re-isolated from vaccinees and experimentally infected monkeys showed plaques indistinguishable from those of the infecting strain in each case, suggesting that one further cycle in vivo had not altered this property at least.

A range of plaque sizes were encountered in the laboratory adapted strains examined, the three attenuated strains RA27/3, Cendehill and HPV-77 producing small, intermediate and large plaques respectively. These results coupled with the findings of large and small plaques among the low pass strains would seem to suggest that there was no general relationship between plaque morphology and attenuation.

For only two of the low pass strains was there any available evidence of virulence, the Dunning strain being implicated in a laboratory case of rubella and the Sheppard strain shown to be virulent for monkeys on the basis of the criteria laid down by Parkman et al (1969) in that there was an extensive excretion of virus from the infected animals, (Draper et al 1969). These two strains produced large and small plaques respectively, again implying the lack of a general correlation between plaque size and virulence.

The Judith strain A was found to give a small plaque at the third pass in rabbit kidney whilst Judith (B) at the fiftieth passage level in GL-RK_{13} gave large clear 2.5-3.00 mm. plaques, thus supporting the suggestion of Morgan (1969) that passage of this strain in GL-RK_{13} resulted in an enlargement of the plaques produced. Similarly the Giguerre (1) strain at the second passage in monkey kidney tissue gave characteristic 1.00 mm. plaques whereas Giguerre (2) after seven further passages in GL-RK_{13} produced a mixed population of small and
large 2.0–2.25 mm, plaques possibly suggesting that at least a proportion of plaques were increasing in size on passage. Further passage of both the samples of this strain are necessary before one can be certain of the effects of passage, it may well be that the mixed plaque population was an intermediate stage in the evolution of a large plaque type.

It was only possible to demonstrate a change in plaque morphology on limited passage of four strains of rubella virus. The Dunning and Janine strains produced smaller plaques after passage in monkey kidney tissue, as did the Janine strain after passage in GL-RK₁₃ cells, whilst the Cendehill and Thomas strains showed an increase in plaque size after passage in BHK-2₁ cells. In none of the other low pass or laboratory adapted strains could a change in plaque morphology in GL-RK₁₃ cells be demonstrated. One might expect that the laboratory adapted strains would have a stable plaque character however this was obviously not the case with the Cendehill strain. Oxford (1969) has reported that freshly isolated strains of rubella show a marked increase in the extent and rapidity of appearance of cytopathic effects in GL-RK₁₃ cells on passage in this cell system, and one might expect to see this expressed as an increase in plaque size, however this could not be demonstrated in any of the strains examined after passage in GL-RK₁₃ cells. Only the Dunning and Janine strains passed back through GL-RK₁₃ cells from the fifth level in monkey kidney tissue showed an increase in plaque size. There did not appear to be any general relationship between passage and plaque morphology, and the effects of tissue culture passage of a strain of rubella may well depend on the tissue selected, the previous history of the strain or the particular properties of the strain examined. Morgan (1969)
was similarly unable to find a clear relationship between the plaque
type and passage history of strains he examined. Wasington et al
(1969) had reported that the change in HFV strain plaques from small
to large occurred in the region of the 27th passage level and it is
possible that further passage of the low pass strains examined in this
study will be necessary before alterations in plaque morphology will be
found. It may be that the strains showing changes in plaque characteristics
after limited passage such as the Giguerre, Dunning, Janine, Thomas and
Cendehill strains are in fact the exceptions and much more extensive
tissue culture passage is generally required before an alteration in
plaque morphology is seen. It would seem that the type of change seen
with the Cendehill and Thomas strains was not unexpectedly an adaptation
process, the plaques gradually becoming larger with passage, whilst that
seen in the Janine and Dunning strains was more similar to a selection
process, a small plaque type appearing only in passage and rapidly
becoming predominant with the loss of the large plaque type. It was
perhaps a little difficult to understand why the small plaque type did
not appear in these latter strains prior to passage if it was present
to be selected under the pressure of tissue culture passage, however it
was possible that a certain percentage of the plaque population was very
rapidly altered from large to small within the space of 2 passes without
going through the intermediate levels, this new plaque then grew well
and the large type was lost either by the selective pressure of the
tissue passage or because it too converted to the small plaque type.

It was interesting that the Janine strain was apparently more
readily altered as regards plaque morphology than was the Dunning strain,
being reduced in size by passage in monkey kidney and GL-RK. This
might be explained by two possibilities; firstly the Janine isolate may
not have been derived from Dunning and the circumstantial evidence implicating the Dunning strain in this laboratory case was purely coincidental, or secondly, and of much greater interest, the passage in vivo had altered the Dunning strain in some manner rendering it more likely to change its plaque character on passage. No change in the plaque characteristics of strains isolated from vacinees or animals has been demonstrated, however these have not been subjected to more than 2 passages in tissue culture after re-isolation and it was possible that further passage might result in the emergence of some detectable alteration in this property.

Cloning of plaques from the mixed population of the intermediate passage levels of Dunning and Janine and from the Giguere (2) strain may yield interesting results. Cloning techniques have not proved successful in this investigation and it was felt that limit dilution methods were too inaccurate to be certain of true cloning.

It was possible to demonstrate the difference between strains at the two extremes of plaque size, in GL-RK₁₃ under fluid maintenance medium, suggesting that an increase in the extent of cytopathic effect is expressed as an increase in plaque size. Fogel et al (1969) have reported that the variations in the plaque morphology in GL-RK₁₃ of the strains they examined were lost if the plaque test was carried out under a carboxymethyl cellulose overlay, and they suggest that these findings indicate that such variations are in fact a reflection of the varying sensitivity of different strains to inhibitors present in the agar. The differences noted above, under fluid maintenance medium in the absence of agar, would suggest that this is not wholly the reason for variation in plaque size between strains, and furthermore the lack of such variation under the same batches of agar in the Vero plaque test would
It is interesting to note that some strains of Vero cells have been reported to lack the ability to produce interferon \cite{Desmyter1968, Kohno1972} and whilst the particular strain utilized in this study has not been tested for interferon production, it was found that rubella virus did not interfere with superinfection of these Vero cells with Newcastle Disease Virus \cite{Gould1968}. It may well be that the variation in plaque size found between some strains of rubella is due in part at least to differences in sensitivity and induction of interferon in GL-RK_{13} cells, certainly the influence of such factors has been demonstrated very neatly for Seniiki forest virus, Newcastle Disease virus and Vesicular Stomatitis virus in L cells \cite{Pauconnier1970} and Mirchamsy & Rapp \cite{1969} have shown that differences detected between the plaques produced by certain strains of measles virus in BSC-1 cells are not found in Vero cells and that this is due in part at least to interferon production.

Thus a range of plaque sizes in GL-RK_{13} cells have been found amongst the low pass and laboratory adapted strains. The majority of low pass strains were found to give small 1.00 mm plaques but two unusual strains were encountered which produced large clear plaques at a low passage level. There did not seem to be any general relationship between the plaque type of a strain and its previous history and the majority of the strains examined could not be altered by limited passage. Where alterations in plaque size were found to occur on limited passage they seemed to take place by two apparently different processes. Some evidence was found suggesting that prolonged passage could increase the size of plaques produced by certain strains. Extremes of plaque type were found to be expressed by small differences in the cytopathic effect produced in GL-RK_{13} cells under fluid medium, but no variation in the plaque size produced in Vero cells could be found between the strains examined.
SECTION III

The growth characteristics of selected strains of virus in varied culture systems.
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Although many cell and tissue culture systems have been studied for the growth of rubella virus and its antigens, such as those described by Yaheri, Sedgwick and Plotkin (1967) and Fabiyi, Sever, Ratner and Caplen, (1966), Leerhoy (1968) and Parkman et al (1969), few reports have been concerned with a comparative examination of the growth characteristics of varied rubella virus strains. Several authors have examined a particular system for titration or growth of several strains, (Fucillo, Gitnick, Traub, Wong, Sever and Hübner, 1967, Liebhaber, Riordan and Horstmann, 1967) but these have largely dealt with one or two strains at different passage levels. Direct comparisons of the growth curves and antigen production of low pass strains have apparently not been undertaken, and where any comparison of such strains have been made they have usually been based on the extent and time of appearance of cytopathic effects (Weller et al, 1962, Reddick et al, 1966, Oxford, 1969). Grayzel and Beck (1971) compared the growth curves of attenuated strains of rubella in human synovial cells and were able to find differences in the production of virus with time. In view of such findings, it seemed possible that similar examination of the growth characteristics of representative strains of rubella might yield interesting information as to the influence of the previous history of the virus and perhaps differences in the basic growth properties of low pass strains. Variations in the size of plaques produced by different strains in GL-RK_{15} cells had been found which might be due in part to variation in the growth properties in this cell system, thus it seemed worthwhile to examine the growth curves of these strains in this and other cell systems.
In order to undertake such investigations it was necessary to lay down large working pools of each strain from which samples could be drawn for each experiment. It was intended that some serological and immunological studies would also be carried out, therefore it was necessary to produce pools of haemagglutinating antigen for each strain. It was desirable that the passage level of each strain be kept as low as possible in order to minimise the possibility of some change in property occurring on repeated culture, and furthermore that the treatment of each strain be kept as similar as the attainable titres would allow. In order to determine how readily adequate titre pools of virus and haemagglutinin for each strain could be produced in cell systems reported to give good titres of both, a comparative study of the yields of each strain was undertaken in BHK21/13S cells (Halonen, Ryan & Stewart 1967) and Vero cells (Liebhaber et al 1969), this served to produce information on how readily each strain adapted to the new cell system and reached maximum output of virus.

GL-RK\textsubscript{13} and BHK21/13S cells were selected as being representative of the cultures generally in use in rubella laboratories for titration or production of rubella virus and its antigen. McCoy cells are derived from human synovial cells, and were included in order to determine whether the results obtained in this system would be similar to those of Grayzel et al (1971) in fresh synovial cells.
1) Strains of virus selected for study

The strains selected for study in this section were chosen as being representative of three categories of rubella; attenuated, laboratory adapted strains; low pass strains from pre-natal infection and low pass strains isolated from post-natal infection. These representative strains are listed in Table 15 with details of their previous history; the titres and passage levels in Vero cells at which pools were laid down are also included. Samples for all growth curve estimations were drawn from pools which were held at -70°C.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>PREVIOUS HISTORY</th>
<th>PASSAGE LEVEL IN VERO + TITRE AT WHICH POOL WAS LAID DOWN</th>
<th>HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA27/3</td>
<td>PASS 30 in WI-38</td>
<td>PASS 2 at 5.45pfu/0.5ml</td>
<td>64 HAU</td>
</tr>
<tr>
<td>HPV-77</td>
<td>PASS 78 in MK*</td>
<td>PASS 5 at 6.20pfu/0.5ml</td>
<td>128 HAU</td>
</tr>
<tr>
<td>CENDEHILL</td>
<td>PASS 53 in YRK†</td>
<td>PASS 3 at 6.50pfu/0.5ml</td>
<td>64 HAU</td>
</tr>
<tr>
<td>DURNING</td>
<td>PASS 6 in GL-RK₁₃</td>
<td>PASS 3 at 4.65pfu/0.5ml</td>
<td>32/64 HAU</td>
</tr>
<tr>
<td>THOMAS</td>
<td>PASS 3 in GL-RK₁₃</td>
<td>PASS 2 at 4.65pfu/0.5ml</td>
<td>128 HAU</td>
</tr>
<tr>
<td>LESLEY</td>
<td>PASS 1 in MK</td>
<td>PASS 2 at 4.40pfu/0.5ml</td>
<td>32/64 HAU</td>
</tr>
</tbody>
</table>

* Monkey kidney tissue
† Primary rabbit kidney tissue.

Table 15. Titre and passage history of the strains of rubella virus selected for study.

Production of these working pools is described in detail in Section I
2) Assay of haemagglutinin

The method of Stewart et al (1967) was adopted, with pigeon erythrocytes being used in place of one day old chick cells. Samples for assay were serially diluted in two-fold steps in dextrose gelatin veronal buffer in microtitre trays with the aid of 0.025 ml. microdiluters and 0.025 ml. droppers.

The blood was freshly collected from birds into Allsevers solution washed three times with 0.85% saline and made up to a 0.2% concentration in dextrose gelatin veronal buffer. The red cell suspension was kept at +4°C and never retained longer than 48 hours. Haemagglutinin titrations were incubated at 36.5°C for 60 minutes or more usually at +4°C overnight, in the latter case trays were held at room temperature for 30 minutes before being read. The titre of antigen quoted was the reciprocal of the highest dilution showing 50% agglutination of the erythrocytes. Two titrations of each sample were carried out in any one test, together with a titration of a freeze-dried standard control haemagglutinin produced by Wellcome Reagents Ltd. All microtitre equipment was manufactured by 'G3CO'. 
3) Assay of infectivity

All assays of rubella virus strains were carried out in the modified GL-RK<sub>13</sub> plaque test (See Section I). Virus dilutions were made in buffered Eagles MEM and 3 plates were inoculated with each dilution. The standard control viruses S1 (RA27/3) and ARl (HPV-77) were included in every test. Titres based on the average plaque count per sample were expressed as log<sub>10</sub> pfu/0.5ml.
4) Method for estimating the rate of adaptation to growth in BHK21/13S and Vero cells.

Confluent 3-day old cultures of BHK21/13S or Vero cells were drained and washed three times with serum free maintenance medium. Each of 2 cultures (4oz medical flat bottles) received a standard inoculum of one of the 6 strains under study. Adsorption was allowed for 30 to 45 minutes at 36.5°C before the cultures were refed with serum free maintenance medium, one further medium change being carried out 24 hours later. Fluids were harvested on the 5th day and 1.0ml volumes were immediately passaged to a further batch of cultures, the excess fluids were stored in 1 ml. volumes at -70°C. Samples of the fluid harvest were titrated for viral content in the GL-RK^13 plaque test. The cells from infected bottles were scraped into 0.5ml of the fluid medium, an equal volume of glycine buffer at pH10 was added and the whole was set at 36.5°C overnight. At the end of this period the cell suspension was sealed tightly and subjected to 3 cycles of freezing and thawing in an alcohol-CO₂ bath. The samples were titrated for haemagglutinin content and stored at -20°C. The process was repeated at each passage with a constant 1.0 ml. volume of inoculum. Harvests from all passage levels were retained at -70°C for further use.
5) Method for estimation of the growth rate of virus in various culture systems;

A) GL-RK₁₃

Test tubes cultures of GL-RK₁₃ cells were prepared as described in Section II. The cultures were changed to fresh MM and each was inoculated with approximately 1000pfu of the selected strain of rubella virus; all strains under investigation being included in one experiment. Rolled incubation was carried out at 36.5°C. Harvests were taken daily from Day 2-7, the fluid from 3 tubes being pooled and the cell sheets washed 3 times with fresh MM before being scraped into a further 1ml. of fresh MM and disrupted by 3 cycles of freeze/thawing. Harvests were either titrated immediately or stored at -70°C until infectivity estimations were possible.

B) Vero

The method was as described above for GL-RK₁₃ cultures.

C) BHK21/135

The method was as described above for GL-RK₁₃ cultures, except that harvests were only taken up to Day 5, at which time the condition of the cell sheets deteriorated rapidly.

D) McCoy

The method was as described above for GL-RK₁₃ cultures.
RESULTS

1) The rate of adaptation of selected strains of virus to growth in BHK21/13S cells.

Figure 32 illustrates the titres obtained at the first passage and at each successive level after further blind passage, up to the tenth harvest. Infective titres were assayed from pass one to four, and haemagglutinin from pass one to ten.

The RA27/3 strain was apparently well adapted to this cell system and grew well immediately, showing significant HA and an increase in virus infectivity titre on first passage, rising steadily over subsequent passes. The HPV-77 and Lesley strains grew very poorly, producing only a trace of HA at the third and fourth passage levels respectively with little detectable virus. The remaining strains, Cendehill, Thomas and Dunning adapted well to the cell system with Thomas reaching high yields of virus after 3 passages. The production of haemagglutinin was apparently a good indicator of the adaptation of the strain to the cell system and assays of HA alone were continued to the tenth passage level. As the strains became adapted to the BHK-21 cells with succeeding passage the difference in yield from RA27/3 as compared to Dunning, Thomas and Cendehill were lost, the HPV-77 and Lesley strains did not adapt to the system.

At the 7th passage an error in technique occurred which led to the assay of the alkaline extract for HA prior to cell disruption, it was noted that there was apparently no HA present in the Cendehill infected cell suspension. The samples were subjected to three cycles of freezing and thawing and retested - a titre of 32 was recorded for the Cendehill HA, the other strains showed small rises in HA titre in the range of 2 to 4 fold, harvests from the remaining experiments were tested before and after cell disruption, all the strains except
Figure 32. Virus and haemagglutinin yield on passage in BHK21/13S cells.

HA TITRE OF HARVEST AT EACH PASSAGE LEVEL

VIRUS

HA BEFORE DISRUPTION

PASSAGE LEVEL IN BHK-21/13S CELLS

LOG_{10} pfu/0.5ml.
Cendehill exhibited the characteristic 2 to 4 fold rise in titre, however little or no HA could be found in the Cendehill preparations prior to rupture of the cells, after which treatment titres of 16 to 64 could be obtained.
2) The rate of adaptation of selected strains of virus to growth in Vero cells.

No striking differences were noted between the strains examined, not unexpectedly the HPV-77 strain yielded the highest titres of virus and HA in the first two or three passages, however this strain had had a prolonged passage history in monkey kidney tissue culture. Dunning similarly exhibited a good titre of virus in the first harvest but growth was not sufficiently good enough to produce detectable levels of HA before the fourth level. The remaining strains of rubella showed a gradual increase in virus yield with HA appearing between the 2nd and 4th passage. The Lesley strain produced titres lower than the other strains but reached similar levels of HA beyond the 4th passage level. A similar investigation of HA titre before and after cell disruption was carried out and once again the Cendehill antigen was significantly lower prior to cell rupture. The titres of virus and haemagglutinin obtained are shown in Figure 33.

It was decided that the working pools of these 6 strains would be produced in Vero cultures, a system in which all the strains grew to comparable titres after limited passage. The seeds for production of these pools were in each case the first harvest from the experiment described above, at which titres in the region of 10,000pfu/0.5ml had been obtained. It was found necessary to continue the Cendehill and Dunning passage through one further level due to a lower yield than expected, the HPV-77 seed selected was in fact from the fourth passage level due to a technical error. Full details of the production of the working pools are given in Section I.

Production of haemagglutinin proved to be more difficult and all but the HPV-77 strain had to be taken at least one passage further to obtain adequate titres.
Figure 33. Virus and haemagglutinin yield on passage in Vero cells.
3) The growth rate of selected strains of virus in

A) GL-UK

The growth curves obtained for each strain are shown in Figure 34, where the yields for intra and extracellular virus are shown. However, it must be noted that throughout this section these terms in fact only distinguish virus present in the fluid harvest (extracellular) and in the cell harvest after disruption (intracellular). Maximum titres for intra and extracellular virus were reached by the third or fourth day with a gradual fall-off thereafter. The greatest divergence of the curves for intra and extracellular virus were seen in the Cendehill, Thomas and Lesley strains and the highest titres achieved were with HPV-77.

B) Vero

Figure 35 illustrates the growth curves obtained in Vero cells. Maximum titres were reached by the fourth and fifth day, the intracellular virus reaching a peak 24 hours before the extracellular virus for HPV-77, Cendehill and Dunning. The Lesley and RA27/3 strains produced maximum titres approximately ten-fold lower than the other four strains. There was apparently no significant variation between intra and extracellular virus for any of the strains examined.
Figure 34. Growth curve of intra and extra-cellular virus in GL-RK13 cells.

RA27/3

HPV-77

CENDEHILL

DUNNING

THOMAS

LESLEY

\[ \text{LOG}_{10} \text{ pfu/0.5mls.} \]

\[ \text{DAYS POST INOCULATION} \]
Figure 35. Growth curve of intra and extracellular virus in Vero cells.
The growth curves obtained for RA27/3, Cendehill, Dunning and Thomas are shown in Figure 36, the HPV-77 and Lesley strains did not produce detectable levels of virus. Maximum titres of virus were reached at 4 days for all but the RA27/3 strain, followed by a maintenance or drop in titre on the final day of harvesting. The virus yield of the RA27/3 strain was still rising on the final day in spite of the poor quality of the cell sheets by this time. The highest titres were obtained for RA27/3 and Thomas and again, the Cendehill strain showed a divergence between extra and intracellular virus, the latter being approximately 0.5 log higher at the peak of virus production. The remaining three strains showed higher extracellular virus titres throughout, the difference being most marked for RA27/3 and Thomas on the final day of testing when the cell sheets were beginning to deteriorate.

d) McCoy cells.

The growth curves of all six strains are shown in Figure 37. Little or no growth occurred with the RA27/3 or HPV-77 strains, virus reaching detectable levels in few of the harvests. Cendehill and the three low pass strains showed production of virus with maximum levels between 2.0 and 3.0 logs. Cendehill, Dunning and Lesley showed peak titres at Day 5 with fall-off thereafter whilst the Thomas strain reached the highest levels at day 3 and 4 with a gradual fall off to day 7. The Lesley strain did not produce detectable virus after the fifth day.
Figure 36. Growth curve of intra and extracellular virus in BHK21/12S cells.

RA27/3

HPV-77

- FLUID
- CELLS

CENDEHILL

DUNNING

THOMAS

LESLEY

LOG pfu/0.5ml.

DAYS POST INOCULATION
Figure 37. Growth curve of intra and extracellular virus in McCoy cells.

RA27/3

HPV-77

CENDEHILL

DUNNING

THOMAS

LESLEY

FLUID

CELLS
The simple adaptation experiment designed to ascertain the lowest passage level at which adequate virus and haemagglutinin titres could be obtained, yielded interesting information about the differences in growth characteristics of the six strains examined. The HPV-77 and Lesley strains were unusual in that little or no virus and haemagglutinin antigen was produced in BHK21/13S cell cultures. This finding was confirmed on examination of the growth rate of the Vero-grown pools of these strains in BHK21/13S cultures.

The RA27/3 strain grew well in BHK21/13S when first introduced into those cells, without the normal 'lag' period expected for a virus to adapt to a new culture system. Virus and haemagglutinin were produced on first passage; production of haemagglutinating antigen appeared to be a good indicator of adaptation to a culture system for the majority of strains required two or three passages before significant levels were reached.

The apparent adaptation of RA27/3 to BHK21/13S might well form the basis of a marker system for this strain and certainly the only successful reports of plaque production in this cell system have been with the RA27/3 strain (Plotkin, 1969), a fact which would seem to support the observations noted here. This distinction between RA27/3 and the remaining three strains which grew in BHK21/13S cells was rapidly lost on passage, as the Cendehill, Dunning and Thomas strains reached equal levels of virus and haemagglutinin production. Similarly the lack of growth encountered with the HPV-77 strain may prove to be a useful distinguishing factor, however it must be noted that the low pass Lesley strain also exhibited this property; possibly lack of
growth in BHK21/13S coupled with a further property such as plaque type in GL-RK₁₃ cells may serve the purpose.

No significant differences were noted between the strains in Vero cells, the HPV-77 not unexpectedly in view of its previous history, being well adapted to this culture system.

It was interesting to note that the majority of the Cendehill haemagglutinin was apparently cell bound or masked in some way, for in both BHK/13S and Vero cells haemagglutinating antigen could not be detected prior to cellular disruption. This phenomenon was not observed for the other strains examined. Furthermore only the Cendehill strain showed a higher intracellular virus titre than that obtained for extracellular virus in BHK21/13S growth curve.

The highest peak titre obtained in the GL-RK₁₃ growth curves was that of the HPV-77 strain; with an inoculum grown in Vero this was in fact the only large plaque strain in this experiment, for the Dunning strain was a predominantly small plaque type by the third pass in Vero cells. These results might suggest that the plaque size in GL-RK₁₃ cells may well reflect in part at least the relative efficiency of replication in this cell system. In Vero cultures the RA27/3 and Lesley strains produced marginally lower virus titres. It can be seen that the Cendehill, Dunning and Thomas strains grew as well as HPV-77 in this system, perhaps suggesting rapid adaptation to the same level of replication as HPV-77 with its prolonged passage history in monkey kidney tissue culture.

The marked distinction between RA27/3 and the other strains of rubella examined in BHK21/13S was not entirely borne out in the growth curve experiments. The Thomas strain reached equal levels of virus production which were maintained on the fifth day, however only in the RA27/3 strain
was there a continued rise in both intra and extracellular virus by this point. It may be that passage in Vero has in some way influenced the growth characteristics of the Thomas strain in BHK21/13S cells; certainly this strain appeared to produce levels of virus as high as the laboratory adapted strains in Vero, BHK21/13S and McCoy cells, suggesting possibly a general adaptation to in vitro growth, only in GL-RK\textsubscript{13} cells were significantly lower titres encountered for this strain.

The McCoy cell line was derived from human synovial cells and it was possible that differences in growth characteristics of the six strains of rubella examined, might be related to their ability to replicate in synovial tissues in vivo and hence cause arthritic and arthralgic reactions. RA27/3 and HPV-77 vaccine strains seemed to produce little virus in the McCoy cell system. However the reported incidence of complications after vaccination was lower for Cendehill than for either of these two strains (Cooper, Zering, Weiss, Matters and Krugman, 1969), and Grayzel et al (1971) have reported the production of higher titres of virus with the HPV-77 strain as compared to the Cendehill strain in non-rheumatoid synovial cell strains. These results would suggest that the McCoy cell line does not offer a model system for this type of vaccine reaction, however it was interesting in that it offered another distinction in the growth properties of the RA27/3 and HPV-77 strains as compared to Cendehill and the three low pass strains examined.

Thus, it has been possible to demonstrate variations in the growth characteristics of the six strains of rubella examined in BHK21/13S, GL-RK\textsubscript{13} and McCoy cells which did not appear to be directly related to the previous passage history of the strain, only Lesley of the three low pass strains examined was distinctive in that little or no virus was produced in BHK21/13S cultures.
SECTION IV

The thermal stability of selected strains of rubella virus as measured by surviving infectivity
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Few reports appear in the literature concerning the thermal stability of rubella virus, and those which do appear are largely concerned with vaccine manufacture (Huygelen, Peetermans, Colinet, Zygraich and Fogard, 1969). Kistler et al (1972) have presented results for the Wright strain of rubella but as yet no comparative data is available concerning the effects of heat on strains of rubella virus.

It was decided that a very simple comparison of the 6 strains of rubella under investigation would be made, which would take the form of a study of the fall off of infectivity with time at 50°C and 32.5°C.
1) Strains of virus selected for study.

The strains selected for study in this section are described in detail in Section III and are listed in Table 16 with details of their passage history. All samples employed in this investigation were drawn from the working pools laid down at -70°C - see Section I.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>PREVIOUS HISTORY</th>
<th>PASSAGE LEVEL IN VERO &amp; TITRE OF WORKING POOLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA27/3</td>
<td>PASS 30 in WI-38</td>
<td>PASS 2 at 5.45 pfu/0.5 ml</td>
</tr>
<tr>
<td>HPV-77</td>
<td>PASS 78 in MK</td>
<td>PASS 5 at 6.20 pfu/0.5 ml</td>
</tr>
<tr>
<td>CENDEHILL</td>
<td>PASS 53 in YRK</td>
<td>PASS 3 at 6.50 pfu/0.5 ml</td>
</tr>
<tr>
<td>DUNNING</td>
<td>PASS 6 in GL-RK&lt;sub&gt;13&lt;/sub&gt;</td>
<td>PASS 3 at 4.65 pfu/0.5 ml</td>
</tr>
<tr>
<td>THOMAS</td>
<td>PASS 3 in GL-RK&lt;sub&gt;13&lt;/sub&gt;</td>
<td>PASS 2 at 4.68 pfu/0.5 ml</td>
</tr>
<tr>
<td>LESLEY</td>
<td>PASS 1 in MK</td>
<td>PASS 2 at 4.40 pfu/0.5 ml</td>
</tr>
</tbody>
</table>

Table 16. Passage history and infectivity titres of strains selected for study

2) Assay of residual infectivity

The method employed for assay of the viral content of samples was as described in Section III.
3) Method for estimating thermal stability at 50°C and 32.5°C

Samples of each strain of rubella were drawn from the working pools and diluted to approximately equal titres. 1.0ml aliquots were added to equal volumes of prewarmed buffered Eagles MEM and placed in a water bath at 50°C or 32.5°C. Samples were withdrawn from the sealed bijoux at stated intervals and immediately titrated for infectious virus in the GL-REK plaque test. The surviving virus was quoted as a percentage of the original plaque count estimated at time 0, and the inactivation curves obtained by plotting surviving virus against time.
1) Thermal stability at 50°C

The inactivation curves obtained are shown in Figure 38. Two distinct inactivation patterns were obtained; the two strains Dunning and Thomas gave straight line patterns suggestive of a first order reaction, with virus reaching undetectable levels in 30 minutes. The remaining four strains showed an initial first order reaction followed by a slowing in the rate of inactivation and a tailing effect with complete inactivation after 30 minutes for all but the Cendehill strain. The fastest initial rate of inactivation was recorded for the Cendehill strain, closely followed by RA27/3 and HPV-77, then the Lesley strain and finally Dunning and Thomas.
Figure 38. Percentage plaque survival with time at 50°C.

- RA27/3
- HPV-77
- CENDEHILL
- DUNNING and THOMAS
- LESLEY
RA27/3 and Dunning strains only were examined in this section. The inactivation patterns obtained are shown in Figure 39. A simple straight line inactivation pattern was found for the Dunning strain, whilst that recorded for the RA27/3 strain was still a two part curve with slowing in the rate of inactivation occurring. It was interesting to note that the rate of inactivation of the Dunning strain was faster than that of RA27/3 at this temperature.
Figure 39. Percentage plaque survival with time at 32.5°C.
Two distinct types of inactivation pattern were found, the two congenital strains Dunning and Thomas showed a typical first order reaction with reduction of virus to undetectable levels after 25 minutes, the remaining strains exhibited a tailing effect, some with incomplete inactivation at 30 minutes. Kistler et al (1972) have reported a similar effect with the Wright strain of rubella, showing a fall off followed by a surviving population even after 30 minutes at a temperature of 70°C; they suggest that the phenomenon may be due to a screening effect of cellular debris present in the virus pool, for total inactivation can be achieved if the sample is filtered through a 0.22 membrane prior to inactivation, or heating continued to 120 minutes. It is possible that viral aggregates might also have the effect of screening some of the population from the thermal radiation, resulting in a deviation from the first order curve. Albrecht & Schumacher (1972) have interpreted similar two part curves for measles virus as being indicative of a heterologous population, with the straight line, first order pattern, implying a homologous virus suspension.

However Woese (1956) in a survey of thermal inactivation of viruses, noted that the two part curve, deviating from the simple exponential law, is in fact the normal result with most animal viruses.

At first sight it would appear that an extrapolation of the second part of the curve would indicate the percentage of the population being inactivated at a slower rate, however this percentage alters with the temperature of inactivation, becoming almost 100% with some viruses. (Kaplan, 1958, Woese 1956). In order to determine whether this was the phenomenon seen in rubella virus, two of the strains were sampled
the intercept was found to have moved towards 100%, as described by Kaplan for vaccinia virus (1958), thus suggesting that the two part curves seen for rubella virus are not explained simply by heterogeneity; the first order line for Dunning remained unchanged. Hiatt (1964) has suggested that the two part curve may be explained by the presence of both intact and uncoated infectious virus, the latter being freed by heat denaturation of the protein coat, thus the heterogeneity is in fact induced by heat input. It is possible that the uncoated virus would be less efficiently detected in an assay system than complete virus and whilst the rates of inactivation i.e. the slope of the two parts of the curve, would remain unchanged, the intercept found on extrapolation might differ in an alternative assay system. Hovi et al (1970) have shown that uncoated rubella particles are still infectious and it remains possible that Hiatt's theory may apply to rubella virus. It thus became possible that the four strains showing two part curves were varying in their sensitivity to the heterogeneity-inducing effect of heat, resulting in variation between the extrapolation intercepts, as well as a simple variation in sensitivity to thermal inactivation.

It is difficult to interpret the straight line, first order results for Dunning and Thomas beyond an assumption that they differ in some physical property from the other strains examined which renders them more resistant to the uncoating potential of the heat input. It may well be that at higher temperatures a two part curve would be seen, for it must be noted that for vaccinia virus (Kaplan 1958) the curves were approaching linearity by a temperature of 50°C. It is interesting that both of these strains originated from congenitally infected infants and it is possible that prolonged replication in vivo
has resulted in this alteration in property.

Considering the only initial parts of the inactivation curves for these 6 strains of rubella, it becomes apparent that the sensitivity to thermal inactivation lies in a range from Dunning and Thomas, apparently most stable, through Lesley to RA27/3 and HPV-77 and finally to Cendehill showing the greatest heat lability.

The change in the relative stability to inactivation at 32.5°C was interesting, with the initial rate for RA27/3 being slower than that of the Dunning strain. These results may well suggest that the differences noted between the strains at 50°C may be more than a simple graduation in stability to thermal inactivation. A comparison of the inactivation profiles of all the strains at several temperatures might well prove interesting, however the technique employed here would probably be too crude for such comparisons, for the accuracy of temperature maintenance in a simple bench water-bath would not be sufficiently fine for comparisons to be made over a close range of temperatures.
SECTION V

Immunogenicity of rubella virus in rabbits and a comparison of selected strains by haemagglutination inhibition and neutralization tests.
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Discussion 184
Huygelen and Peetemans (1967) reported the apparent inability of the Cendehill strain after extensive rabbit kidney passage, to induce antibodies in rabbits. Oxford (1969) however was able to demonstrate a low rate of conversion using multiple inoculations and a different strain of rabbits. Furthermore, Oxford was able to show a strain-related graduation of response, with rabbits receiving low pass strains exhibiting conversion to higher neutralizing titres of antibody. Similarly, London, Fucillo, Ley and Sever (1971) found that a single intravenous inoculation elicited a response which clearly differentiated between wild and attenuated strains. Zygraich, Peetemans and Huygelen, (1971) were able to show that intravenous inoculation of Cendehill virus could induce an antibody response in rabbits, whilst subcutaneous and intradermal administration failed to do so in the five hundred animals tested. Oxford and Potter (1970) had also demonstrated the failure to induce Cendehill antibodies using unpassaged vaccine administered intramuscularly. They also reported that two inoculations of 1000TCID$_{50}$ of wild virus were required to induce a good antibody response, whilst Zygraich et al (1971) achieved similar levels after administration of as little as 5 TCID$_{50}$ of such strains. It seemed possible that the passage of even the wild strains through rabbit tissue prior to inoculation, as carried out by Oxford et al (1970), might well reduce the immunogenicity of such strains in the rabbit. Thus in the light of these data it was decided that rubella virus grown in tissue other than rabbit would be most effective in inducing antibody and furthermore that the intravenous route would also prove more rewarding. Two experiments were carried out, the first in which Californian strain rabbits received weekly inoculations of the six strains of rubella virus by the intravenous route and the second in which a single inoculation was administered by the same route.
Banatvala et al (1969) and Best and Banatvala (1970) were not able to demonstrate a significant variation in the strains of rubella they examined by standard cross or kinetic haemagglutination inhibition techniques, similarly Kono (1969) was unable to show any antigenic difference between Japanese strains and American strains of rubella in a simple cross HAI test. It was not possible to apply a kinetic HAI to the sera obtained in this study as the titres of haemagglutinin were not adequate for all of the strains, and it was felt that passing virus to higher levels to obtain the required titres would introduce an unwanted variable into the procedure, for it was not known how a strain might alter in antigenicity with passage. Certain properties are known to change on passage, and all strains may not be affected in the same way, for example, antigenicity in rabbits, plaque formation and growth potential in certain tissues, (Oxford 1969, Morgan 1969, Gould et al, 1972).

It was thought desirable to keep the passage level of each strain as low as possible in order to minimise variation from the original material and also to keep the passage histories of the strains as similar as possible once they had been received in the laboratory. Furthermore with regard to tests involving HA it was decided that a whole virus haemagglutinin as produced by alkaline extraction, possibly provided a more sensitive indicator of antigenic variation than an ether split antigen. In using the same antigens for induction of antibody and its assay, a totally homologous system could be ensured; however in order to obtain sufficient titres of haemagglutinin the antigen generally received one further passage on from that used for inoculation of the rabbits.
most sensitive in any system for detection of serological variation
(McBride 1959, Best et al, 1970), further Saturno and Henderson (1965)
and Young and Johnson (1969) have shown that antisera against Western
equine encephalitis and Venezuelan equine encephalitis respectively,
could vary in their specificity depending on the animals and the
immunizing programme adopted. It was decided that a complete cross
haemagglutination inhibition examination of all the bleedings from
rabbits given single and multiple immunizing doses would cover the range
from early to hyperimmune sera, and include animals on exactly similar
immunizing programmes; further variations in the response pattern of
the animals when assayed against heterologous antigens might indicate
some strain variation.

The only reports of comparative studies of strains of rubella virus
in the neutralization test have involved an assay of the residual virus
surviving neutralization by rising dilutions of antisera. Plotkin et al
(1969) and Oxford (1969) were able to show small serological differences
between the strains they investigated, and furthermore Plotkin was able
to demonstrate the existence of a particularly antibody-sensitive strain,
HPV-77, which was always neutralized more effectively than the other
strains examined, by both homologous and heterologous antisera.
Kono (1969), however was unable to show any differences in the strains
he investigated by simple estimation of the antibody titres against
Japanese and American strains of rubella virus.

McBride (1959) had been able to demonstrate small antigenic
differences within the polio virus types by measurement of the velocity
of the neutralization reaction of a particular antiserum against
homologous and heterologous strains of virus, he noted that the serum
neutralized the homologous virus at a faster rate than the heterologous
(Adams, 1950; Luria, 1953), and required only that the neutralization reaction proceeded by first order kinetics, initially at least. Dulbecco, Vogt and Strickland (1957) were able to demonstrate that the kinetics of neutralization of animal viruses could in some cases be similarly analysed and the rate constant (K) of a given serum-virus neutralization reaction calculated. Comparisons of the rate of neutralization or the rate constant, K, could then be made for one serum against several antigens, and minor variations highlighted, which were too fine to be detected by other techniques. Although Rawls, Desmyter and Helnick reported a study of the neutralization process of rubella virus in 1967 and were able to show that it proceeded by first order kinetics over the first 15-20 minutes of reaction time, little further work has appeared on this aspect of rubella serology since that time. Many workers have been hampered by the lack of a sufficiently sensitive titration technique for estimation of residual virus, those who have had such a technique have investigated the factors affecting the reaction, such as the addition of fresh non-immune serum to heat inactivated antiserum-antigen mixtures (Taylor-Robinson and Ratcliffe, 1969).

Thus, in the light of existing data suggesting the possibility of small serological differences between strains, a brief investigation of the rates of neutralization in homologous and heterologous systems was undertaken. It was first necessary to examine the neutralization reaction by the plaque reduction test in order to determine whether it followed first order kinetics and if so under what conditions would estimations of the rates of reaction be optimal.
1) **Strains of rubella virus.**

The strains of rubella virus selected for investigation are described in detail in Section III.

All materials for the inoculation of rabbits were drawn from these pools as were the RA27/3 virus and strains of haemagglutinin for antibody assay.
Two separate experiments were carried out in the Californian strain of rabbits; the first in which repeated inoculations were given and the second in which a single inoculation of the same material was administered. The animals received 5.0 ml. volumes of inoculum via the ear vein either on a single occasion or at stated intervals. Two rabbits were included for each strain in the multiple inoculation experiment and one rabbit per strain in the single inoculation experiment. 5.0 ml. sample bleedings were taken from the ear vein according to the schedule outlined below, beginning on Day 0, prior to inoculation. Blood samples were held at 37°C for 60 minutes or at +4°C overnight, the sera separated off, clarified and stored at -20°C in 0.2 ml. aliquots. At the end of the experiment the animals were anaesthetised and bled out from the heart.

Rabbits receiving low pass virus were housed separately from those receiving vaccine strains in order to avoid cross infection. Uninoculated rabbits were included in each group as a further check of the possible spread of infection - at no time did these animals show a detectable level of neutralizing or haemagglutination-inhibiting antibody to rubella.
A) Treatment of sera.

0.1 ml. of serum was added to 0.9 ml. of 0.85% Saline and inactivated at 56°C for 30 minutes. The product was cooled to room temperature before the addition of 0.05 ml. of Heparin/manganous chloride solution, (Feldman, 1968) consisting of Heparin at 4000 units/ml and Manganous chloride as a 1 Molar solution. The sera were set at +4°C for 20 minutes before being centrifuged to remove any precipitate which had formed. In order to remove non-specific agglutinins, 0.05 ml. of a 50% suspension of pigeon erythrocytes in DGV was added to each serum sample and the whole left at +4°C for 60 minutes, before being centrifuged, once again, to remove the red cells. Treated sera were usually stored overnight at +4°C before being used in the HAI test.

Dextrose gelatin veronal buffer (DGV) was used throughout the test as diluent. (Appendix). 0.20% pigeon erythrocytes formed the red cell system, these having been collected in Allsevers and washed three times in 0.85% Saline before being made up to percentage in DGV.

Cooke microtitre plates were used throughout.

B) The haemagglutination inhibition test.

Antisera were used at 1/10 and 2 fold dilutions were made from this point with the aid of 0.025 micro-dilutors (Cooke Engineering Co.). Four HA units of each antigen were added to each of the serum dilutions, the HA titres having been estimated immediately prior to use in the HAI test. (see Section III). The antigen-antibody mixtures were incubated at 37°C for 20 minutes before the addition of the red blood cells, after which incubation was carried out overnight at +4°C. The plates were held at room temperature for 30 minutes before reading. Serum titres were read as the highest dilution showing complete
cell controls, titrations of the antigen employed, and titration of the standard antigen (supplied by Wellcome Reagents Ltd.) and the standard positive and negative sera (Pre and post-immune serum from rabbit R50/70, see Section I).
4) Assay of Neutralizing Antibody

Three, four-fold dilutions of uninactivated serum were mixed with equal volumes of one dilution of RA27/3 virus containing approximately 30-40 pfu/0.1 ml. The mixtures were held at 32.5°C for 60 minutes and residual virus was assayed in the plaque test, each of three plates receiving 0.1 ml. of a particular virus-serum mixture. Non-immune serum controls, together with a standard control antiserum (R50/70, see Section I) of known titre were included in every test.

The percentage plaque reduction calculated by taking the plaque count with non-immune serum as 100%, was plotted against the dilution of antiserum. From this graph the titre of antiserum could be read as that dilution giving a 60% reduction in plaque count.
One or more dilutions of a single antiserum were mixed with an equal volume of virus, the mixture was then incubated in a sealed bijoux bottle in a water bath set at 32.5°C and 0.1 ml. samples were withdrawn at zero time and at stated intervals thereafter. The samples were immediately inoculated onto petri dish cultures of GI-RK13 cells, along with similar mixtures of pre-immune serum with virus and standard control serum with standard control virus.

The surviving virus i.e. the ratio of virus at time t to the virus originally present at time 0, was plotted on a logarithmic scale against time on a linear scale, and the rate of neutralization per minute calculated from the initial slope of the curve where the reaction was apparently first order, i.e. a straight line passing through the origin (Dulbecco et al, 1956). Using the equation (1) Dulbecco et al (1956).

\[
\text{Slope of the curve} = \frac{K}{D} = \text{rate of neutralization/minute} (1)
\]

where \( K \) = the rate constant of neutralization

and \( D \) = Dilution of antiserum

the \( K \) value could be calculated for each antiserum against each antigen.

In order to clarify the results and make comparisons easier, the \( K \) values were normalised (McBride 1959) i.e. the \( K \) value for the homologous serum and virus was taken as 100% and the values for the heterologous system quoted as percentages thereof.

Assay of one serum against all the strains of rubella virus was always carried out in one test to avoid variations between separate experiments. Pools of virus were diluted to approximately equal infectivity titres (± 0.5 log) before mixing with the serum.
1. Haemagglutination- inhibiting and neutralizing antibody response in rabbits assayed against RA27/3 strain antigens after

A) Multiple inoculations

The haemagglutination inhibition antibody levels are shown in Figure 40 and the neutralizing antibody response in Figure 41 (Inoculation and bleeding dates are also indicated). Unfortunately the second rabbit receiving Cendehill antigen died early in the experiment due to an unrelated disorder.

No striking differences in the response to each strain was noted, all of the strains examined induced neutralizing and haemagglutination inhibiting antibody to similar levels, indicating that all the strains tested were immunogenic at that point in their passage history. The highest HAI antibody levels were comparable for each strain, whilst some difference was noted in the levels of neutralizing antibody reached, the highest titres for HPV-77, Dunning and Thomas being between 2 and 5 fold higher than those induced by the RA27/3, Cendehill and Lesley strain. The peak titres reached to the point of bleed out are given in Table 17. The response to the first inoculation was surprisingly rapid with neutralizing and HAI antibodies being present at detectable levels in some of the rabbits at the point at which the second immunizing dose was administered i,e. 7 to 10 days.
Figure 40. HAI antibody response of rabbits after multiple inoculations. Each animal received a further inoculation after a sample bleeding was taken.
Figure 4.1. Neutralizing antibody response of rabbits after multiple inoculations. Each animal received a further inoculation after a sample bleeding was taken.
<table>
<thead>
<tr>
<th>STRAIN OF RUBELLA</th>
<th>PEAK TITRES OF ANTIBODIES AS ASSAYED AGAINST RA27/3</th>
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<tr>
<td></td>
<td>HAI ANTIBODIES +</td>
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<td></td>
<td>MULTIPLE INOCULATION</td>
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<tr>
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**TABLE 17.** Highest neutralizing and HAI antibody levels reached after administration of multiple and single immunizing doses of each strain of rubella virus.
B) Single inoculation

The development of haemagglutination-inhibiting and neutralizing antibody is shown in figure 42 with details of the dates of bleeding.

All the rabbits showed an antibody response indicating that all the strains were immunogenic. Neutralizing antibody was present in significant levels for all the strains up to 50 days after the immunizing inoculation, and all but Cendehill showed HAI antibodies to this point also. Very high levels of HAI antibodies of up to 5120 were seen in the rabbits receiving HPV-77, Dunning and Thomas strains which were of the same order as those noted in rabbits receiving repeated inoculations of these strains, similarly the Lesley strains showed comparable titres after single and multiple inoculations. The rabbit receiving Cendehill antigen showed a lower response after one inoculation than those which had received repeated doses, the highest titres reached for HAI being 160 and 2560 respectively, the RA27/3 also demonstrated a marginally lower response after a single immunizing dose. The HPV-77 and Lesley strains produced neutralizing antibody levels of the same order after single and multiple inoculations, the Lesley response being very similar in both experiments whilst the rise for HPV-77 occurred more quickly after a single immunizing dose. All the other strains investigated showed a lower response after a single inoculation, the differences being in the order of a 2 to 4 fold higher antibody level at the highest peak. Table 17 compares the peak antibody titres found after multiple and single inoculations.
Figure 42. HAI and neutralising antibody response of rabbits after a single inoculation.
2) Haemagglutination-inhibiting antibody response in rabbits assayed against homologous and heterologous antigens after

A) Multiple inoculations

The results of the cross HAI tests are given in Tables 18, 19 and 20. No significant variation in the titres for antiserum as assayed against the six antigens could be found for HPV-77, RA27/3, Dunning, Thomas and Lesley, however unexpected differences were noted for the Cendehill antiserum. It was not possible to detect antibody to the Cendehill antigen in the serum taken on the tenth day, despite the fact that this serum yielded titres of up to 1280 against the remaining 5 antigens. Sera from subsequent bleedings showed no significant variations when assayed against homologous and heterologous antigens.
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**RA27/3 ANTIERA**

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**HPV-77 ANTIERA**

Figure 18. Haemagglutination-inhibiting antibody titre of sera collected from rabbits receiving multiple inoculations.
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**CENDEHILL ANTISERA**

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**DUNNING ANTISERA**

Table 19. Haemagglutination-inhibiting antibody titre of sera collected from rabbits receiving multiple inoculations.
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THOMAS ANTISERA

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<td>42</td>
<td>1280</td>
<td>640</td>
<td>640</td>
<td>320/640</td>
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</tr>
</tbody>
</table>

LESLEY ANTISERA

Table 20. Haemagglutination-inhibiting antibody titre of sera collected from rabbits receiving multiple inoculations.
The results of the cross HAI tests are given in Tables 21 to 26.

No significant variation in antibody titres could be found for HPV-77, RA27/3, Dunning, Thomas and Lesley antisera, the Cendehill antiserum however, was again showing significant differences. Titrations of this serum against Cendehill antigen were significantly lower than those obtained against the remaining strains on day 28, by day 35 the Cendehill titres were lower than those of RA27/3, Dunning and Thomas strains, and by day 50 antibody was not detected against Cendehill, RA27/3 and Lesley antigens.
<table>
<thead>
<tr>
<th>Days Post First Inoculation</th>
<th>RA27/3</th>
<th>HPV-T7</th>
<th>CENDSHILL</th>
<th>DUNNING</th>
<th>THOMAS</th>
<th>LESLEY</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>7</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<td>14</td>
<td>10</td>
<td>10</td>
<td>40</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>21</td>
<td>80</td>
<td>80/160</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>80</td>
</tr>
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<td>28</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>160</td>
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<tr>
<td>35</td>
<td>80/160</td>
<td>80/160</td>
<td>80/160</td>
<td>80</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>42</td>
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<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>80/160</td>
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<td>40</td>
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<td>40</td>
<td>80/160</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>

**RA27/3 ANTISERA**

Table 21. Haemagglutination inhibiting antibody titre of serum collected from rabbits receiving a single inoculation.
### Table 22. Haemagglutination-inhibiting antibody titre of serum collected from rabbits receiving a single inoculation.

<table>
<thead>
<tr>
<th>ANTISERA</th>
<th>HPV-77</th>
<th>RA27/3</th>
<th>CENDHILL</th>
<th>DUNNING</th>
<th>THOMAS</th>
<th>LESLEY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days Post First Inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>&lt;10</td>
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<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
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<td>80/160</td>
<td>80</td>
<td>80</td>
<td>80/160</td>
<td>80/160</td>
<td>160</td>
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<td>320</td>
<td>1280</td>
<td>640</td>
<td>1280</td>
<td>640</td>
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<td>1280</td>
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<td>1280</td>
<td>640</td>
<td>1280</td>
<td>1280</td>
<td>1280</td>
</tr>
<tr>
<td>Days Post First Inoculation</td>
<td>CENDEHILL</td>
<td>RA27/3</td>
<td>HPV-77</td>
<td>DUNNING</td>
<td>THOMAS</td>
<td>IESLEY</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-----------</td>
<td>--------</td>
<td>--------</td>
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<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
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<td>160</td>
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<td>80/160</td>
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<td>80</td>
<td>80</td>
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<td>160</td>
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<td>40</td>
<td>160</td>
<td>40</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

CENDEHILL ANTISERA

Table 23. HAEMAGGLUTINATION-INHIBITING ANTIBODY TITRE IN RABBIT RECEIVING A SINGLE INOCULATION.
<table>
<thead>
<tr>
<th>Days Post First Inoculation</th>
<th>DUNNING</th>
<th>RA27/3</th>
<th>HPV-77</th>
<th>CERDEHILL</th>
<th>THOMAS</th>
<th>LESLEY</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
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</tr>
<tr>
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<td>&lt;10</td>
<td>&lt;10</td>
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<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>14</td>
<td>640</td>
<td>640/1280</td>
<td>320</td>
<td>320</td>
<td>1280</td>
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<td>640</td>
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</tr>
</tbody>
</table>

**DUNNING ANTISERA**

Table 24. Haemagglutination-inhibiting antibody titre in rabbit receiving a single inoculation.
<table>
<thead>
<tr>
<th>ANTISERA</th>
<th>RUBELLA HAEMAGGLUTININ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>THOMAS</td>
</tr>
<tr>
<td>Days Post First Inoculation</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>&lt;10</td>
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<tr>
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</tr>
<tr>
<td>50</td>
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</tr>
</tbody>
</table>

THOMAS ANTISERA

Table 25. Haemagglutination-inhibiting antibody titre in rabbit receiving a single inoculation.
<table>
<thead>
<tr>
<th>Days Post First Inoculation</th>
<th>LESLEY</th>
<th>RA27/3</th>
<th>HPV-77</th>
<th>CERDEHILL</th>
<th>DUNNING</th>
<th>THOMAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<td>640</td>
<td>160</td>
<td>160</td>
<td>320</td>
<td>320</td>
<td>640</td>
</tr>
<tr>
<td>21</td>
<td>640</td>
<td>640</td>
<td>640</td>
<td>1280</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>28</td>
<td>640</td>
<td>640</td>
<td>640</td>
<td>640</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>35</td>
<td>1280</td>
<td>1280</td>
<td>640/1280</td>
<td>640</td>
<td>640/1280</td>
<td>640/1280</td>
</tr>
<tr>
<td>42</td>
<td>2560</td>
<td>1280/2560</td>
<td>1280/2560</td>
<td>1280</td>
<td>1280/2560</td>
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<td>320</td>
<td>320</td>
<td>320</td>
</tr>
</tbody>
</table>

**LESLEY ANTISERA**

Table 26. Haemagglutination-inhibiting antibody titre in rabbit receiving a single inoculation.
Determination of K value of rabbit hyperimmune serum in kinetic neutralization tests against homologous and heterologous rubella antigens.

A) Investigation of the kinetics of rubella virus neutralization in a model system and determination of the optimal conditions for the estimations of K values.

The percentage of plaque survival of SI (RA27/3) virus in mixtures of 4 dilutions of the untreated standard control serum (Edmunds strain, R50/70) was plotted against time on a linear scale - Figure 42. Antiserum at a 1/4 dilution showed a drop in virus to undetectable levels within 5 minutes, whilst the 1/8 dilution gave a rapid fall off over the first 10 minutes followed by a slowing of the reaction with undetectable levels being reached by 30 minutes. At dilutions 1/32 and 1/256 a similar fall off in residual virus was seen at 5 minutes followed by a change in the rate of reaction and what appeared to be grossly abnormal curve. Drops in the amount of residual virus i.e. virus still able to infect GL-RK13 cells, were rapidly followed by apparent increases in the surviving fraction of up to 30%. The experiment was repeated on four separate occasions with a 1/32 dilution of serum in order to determine whether the effect was reproducible, Figure 44. Similar sudden increases of virus occurred at approximately the same points on the curve, although the extent of the rise varied from near the level of significance to almost 40%. Similar experiments were then repeated for antisera to the Thomas, RA27/3 and Cendehill strains against the SI (RA27/3) standard virus, Figure 45, and against other antigens Figure 46. The same effect was seen at the higher dilutions of antiserum in all the reaction mixtures. A similar effect was also seen in mixtures of unheated pre-immune serum and antigen, Figure 47.
Figure 43. The percentage survival of RA27/3 virus in four dilutions of antiserum over a period of sixty minutes.
Figure 44. Percentage survival of RA27/3 virus in a 1/32 dilution of antiserum in four separate experiments.
Figure 45. Percentage survival of RA27/3 virus in a 1/4 and 1/256 dilution of three antisera.
Figure 46. Percentage survival of
(A) Thomas virus in a 1/4 and 1/256
dilution of Thomas antiserum.
(B) Thomas virus in a 1/4 and 1/256
dilution of RA27/3 antiserum.
(C) Cendehill virus in a 1/8 and 1/512
dilution of Lesley antiserum.
Figure 47. Percentage survival of 
(A) S1(RA27/3) standard virus 
(B) RA27/3 virus 
(C) Thomas virus 
in 1/4 dilutions of 3 pre-immune rabbit sera.
Figure 48. Effect of complement on the neutralizing activity of heated and unheated standard antiserum (R50/70) on the standard control virus S1 (RA27/3).
Figure 49. Surviving virus \( S1 (RA27/3) \) in four separate mixtures with a 1/8 dilution of the standard antiserum (R50/70).

\( Vo \) = infectious virus present at time zero.

\( Vt \) = infectious virus present at time \( t \).
The effect was not seen with the antibody to rubella virus when the sera were heated at 56°C for 30 minutes prior to inclusion in the test.

When the complement in the form of fresh guinea pig serum at a dilution of 1/50 was added to mixtures of antigen and heated and unheated sera at dilutions of 1/8 the sudden rises in free virus were not seen. Figure 48. Neither were they seen in mixtures of complement and antigen alone. Addition of complement to the system resulted in an increase in the rate of the reaction.

In four separate tests of the standard unheated serum at a dilution of 1/8 against the SI (RA27/3) standard virus, first order kinetics were seen over the first 10-15 minutes when the surviving virus was plotted against time - Figure 49.

For all further investigations unheated sera at a dilution of 1/8 were employed and K values determined for the first 10 minutes of reaction time. Thirteen separate estimations of the K value for the control rubella antiserum (Edmunds strain) against the standard control pool of virus, SI, (RA27/3) under these conditions, are listed in Table 24.
<table>
<thead>
<tr>
<th>ANTISERUM/ANTIGEN MIXTURE</th>
<th>K VALUE</th>
<th>NORMALISED K VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.66</td>
<td>90.2</td>
</tr>
<tr>
<td></td>
<td>1.96</td>
<td>106.5</td>
</tr>
<tr>
<td></td>
<td>1.86</td>
<td>101.1</td>
</tr>
<tr>
<td></td>
<td>1.98</td>
<td>107.6</td>
</tr>
<tr>
<td>STANDARD CONTROL</td>
<td>1.66</td>
<td>90.2</td>
</tr>
<tr>
<td>EDMUND ANTISERUM +</td>
<td>1.76</td>
<td>95.6</td>
</tr>
<tr>
<td>STANDARD CONTROL</td>
<td>1.94</td>
<td>105.4</td>
</tr>
<tr>
<td>SI (RA27/3) VISIBLE VIRUS</td>
<td>1.66</td>
<td>90.2</td>
</tr>
<tr>
<td></td>
<td>1.78</td>
<td>96.7</td>
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<td>109.2</td>
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<tr>
<td></td>
<td>1.95</td>
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</tr>
<tr>
<td>MEAN VALUE</td>
<td>1.84</td>
<td>99.9</td>
</tr>
</tbody>
</table>

Table 24. K values obtained in thirteen separate estimations of the rate of neutralization of the control antisera/antigen mixture. Normalised K values are calculated from the mean value of K obtained in these experiments. Standard Deviation = ± 7.093
K values were also estimated for the homologous antiserum/antigen mixtures employed in this investigation, all values being obtained within one test. - Figure 25.

<table>
<thead>
<tr>
<th>ANTISERUM</th>
<th>ANTIGEN</th>
<th>K VALUE</th>
<th>NORMALISED K VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA27/3</td>
<td>RA27/3</td>
<td>1.60</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.76</td>
<td>110</td>
</tr>
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<td></td>
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<td>1.44</td>
<td>90</td>
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<tr>
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<td>HPV-77</td>
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<td></td>
<td></td>
<td>2.65</td>
<td>98</td>
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<tr>
<td>CENDEHILL</td>
<td>CENDEHILL</td>
<td>1.56</td>
<td>98</td>
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<td></td>
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<td>1.60</td>
<td>101</td>
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<td></td>
<td></td>
<td>1.63</td>
<td>102</td>
</tr>
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<td>THOMAS</td>
<td>2.46</td>
<td>96</td>
</tr>
<tr>
<td></td>
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</tr>
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<td></td>
<td>2.49</td>
<td>98</td>
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<tr>
<td>LESLEY</td>
<td>LESLEY</td>
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<td>100</td>
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<td>2.94</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.94</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 25. K values obtained within one test for the homologous antiserum/antigen mixtures. Normalised K values are calculated from the mean value of K obtained from the 3 estimations.
B) Determination of the K values of rubella antisera, raised against selected strains of virus, with homologous and heterologous antigens.

The values for the rate constant of neutralization, K, and the normalised K values are shown in Table 26 together with the K values obtained in each test for the standard control antiserum/antigen mixture.

Lower results for the value of K with some heterologous antigens were encountered with each antiserum. Only the HPV-77 and Lesley antigens gave values of K higher than those obtained with the homologous antigen on any occasion.
<table>
<thead>
<tr>
<th>ANTISERA</th>
<th>RA27/3</th>
<th>HPV-77</th>
<th>CENDEHILL</th>
<th>DUNNING</th>
<th>THOMAS</th>
<th>LESLEY</th>
<th>AS.*</th>
<th>AG.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA27/3</td>
<td>1.54</td>
<td>1.66</td>
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<td>0.98</td>
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<td>90</td>
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<td>1.84</td>
<td>0.78</td>
<td>1.04</td>
<td>0.84</td>
<td>1.90</td>
<td>1.96</td>
<td>106</td>
</tr>
<tr>
<td>HPV-77</td>
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<td>1.60</td>
<td>1.48</td>
<td>N.D.</td>
<td>1.86</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>1.55</td>
<td>2.84</td>
<td>1.64</td>
<td>1.60</td>
<td>1.48</td>
<td>N.D.</td>
<td>1.98</td>
<td>108</td>
</tr>
<tr>
<td>CENDEHILL</td>
<td>1.18</td>
<td>2.22</td>
<td>1.68</td>
<td>1.48</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.66</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>1.12</td>
<td>2.04</td>
<td>1.48</td>
<td>N.D.</td>
<td>1.02</td>
<td>2.24</td>
<td>1.84</td>
<td>100</td>
</tr>
<tr>
<td>DUNNING</td>
<td>1.60</td>
<td>1.92</td>
<td>1.68</td>
<td>2.40</td>
<td>0.64</td>
<td>5.04</td>
<td>1.76</td>
<td>97</td>
</tr>
<tr>
<td>THOMAS</td>
<td>1.40</td>
<td>1.60</td>
<td>2.24</td>
<td>1.79</td>
<td>1.98</td>
<td>2.56</td>
<td>1.94</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>1.28</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.92</td>
<td>1.92</td>
<td>2.52</td>
<td>1.96</td>
<td>106</td>
</tr>
<tr>
<td>LESLEY</td>
<td>0.86</td>
<td>3.36</td>
<td>1.60</td>
<td>1.44</td>
<td>1.20</td>
<td>2.88</td>
<td>1.66</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 26. Values of K obtained for one antiserum against all six antigens, within one test, together with the K value obtained for the Standard Control antiserum (Edmund) + standard control virus S1 (RA27/3) in each test. * Standard antiserum and antigen.
It was unfortunate that time and accommodation only allowed one rabbit to be included for each strain in the single inoculation experiment and that only one 'Cendehill' rabbit survived in the multiple inoculation experiment, for it remains possible that any results obtained might be influenced by some factor peculiar to the one animal. It was clear, however, that all the strains examined were immunogenic at that point in their passage history and at the titre administered.

It is interesting to note that in the rabbits receiving vaccine strains the first appearance of detectable antibody occurred later in time in the second experiment in which only one inoculation was given, this is not accounted for by a boost due to the second shot, for at the time of the second inoculation antibody was already present in most of these rabbits. Bleedings were taken at 10-14 days and at 7 days respectively in the first and second experiments, and it is possible that a 3 day difference in time could account for the discrepancy in antibody levels, or it may be that this was merely an instance of variation in response between separate animals for such differences can be seen within the pairs of rabbits in the first experiment, particularly for neutralizing antibody.

Many reports appear in the literature accounting proven replication of low passage rubella in rabbits, (Belcourt et al 1966, London, Fucillo and Sever 1969, London Fucillo, Ley and Sever 1971, Oxford 1969, Kono, 1969). Oxford (1969) has also shown that the high passage attenuated strains can infect rabbits if administered in sufficient titres, and similarly Kono (1969) has shown replication of the apparently ateratogenic Japanese strains. Although re-isolation of virus was not attempted in this study it is possible that an infection of the rabbits
did occur as the antibody titres achieved after a single immunizing
dose of each strain were in most cases equivalent to those found
after a course of inoculations. The RA27/3 and Cendehill strains
may have replicated less efficiently in the animals, if at all,
resulting in the lower titres, certainly with the Cendehill strain
the complete fall off of HAI antibody might suggest that a true
infection had not occurred, however, the neutralizing antibody was
apparently still rising at the end of 50 days. It is perhaps
interesting to note that in the first experiment the haemagglutination-
inhibiting antibody titre for Cendehill and RA27/3 were beginning to fall
in spite of rising neutralizing antibody. It will be necessary to repeat
the experiment including larger numbers of animals which would allow
viral re-isolations to be attempted. It is possible that antigenicity
in rabbits may be of value in distinguishing strains of rubella virus,
however, this may be quantitative rather than qualitative variation
and a single immunizing inoculation may yield more information.

It must be noted that the results in such a survey may well be
influenced by factors such as the rabbit strain, route of inoculation
or treatment of the antigens prior to administration. Certainly,
Zygraich et al (1971) were able to show differences in the response
of rabbits to the Cendehill strain, depending on the route of
immunization. Oxford et al (1970) and Gill and Furesz (1973) were able
to show similar differences in the immunogenicity of the Cendehill
strain after re-isolation from vaccinees, and Gill et al (1973)
suggested that these might be due in part at least, to the isolation
and passaging of the strain in monkey kidney tissue. The high titres
of Cendehill antibody induced in this study may be due to passage in
Vero cells and the choice of route of administration. If it is
of a strain, then the results reported here for the HPV-77 strain which induced higher titres of antibody than the other two vaccine strains may be accounted for by such factors, as may the increased titres of RA27/3 antibody as compared to the results obtained by other investigators such as London et al (1971). The fall off in haemagglutination inhibiting antibody induced with passaged Cendehill strain was also reported by Gill et al (1973), however, the strain of haemagglutinin used for assay is not recorded and neutralization tests were not carried out, therefore a question remains as to whether antibody may have been detectable with other strains or other techniques.

Significant variations in haemagglutination-inhibiting antibody titres when assayed against homologous and heterologous antigens could only be detected in those sera produced in response to the Cendehill strain. These antisera, produced by either single or repeated inoculations of the Cendehill strain, were shown to have a significantly lower titre when assayed against the homologous Cendehill haemagglutinin. Early and late bleedings only demonstrated this phenomenon and it was further noted that the final bleeding from an animal receiving a single inoculation did not demonstrate an antibody titre when assayed with the RA27/3 or Lesley haemagglutinin. It is interesting to note that the Cendehill haemagglutinin differed from that of the other strains in its apparently close cell association, whilst the Cendehill antigen for immunization of the rabbits was fluid derived as were all the other pools. It is possible that small antigenic differences may exist between intracellular virus and extracellular released, virus possibly concerned with acquirement of some factor during or prior to release, thus fluid harvests would contain largely extracellular virus whilst virus obtained by alkaline extraction and cell disruption might be largely cell bound.
Antibody induced by released virus may not be the same as that produced in response to 'cell associated' virus, such a situation might lead to differences in the response in rabbits depending on the immunizing antigen and variation in antibody titrations due to the particular type of antigen selected for the assay system.

Thus the response of a rabbit to Cendehill virus may be influenced by the relative amounts of the two types of antigen in the inoculum depending on the cell system used for its production, and further it might be possible to fail to detect antibody if the wrong haemagglutinin were selected for its assay. The fact that Cendehill has been found to be immunogenic in only a few animals may be a reflection of the difference of immunogenicity of the two components in varied species; it would be interesting to assay these sera against Cendehill HA extracted from the fluid only, without further treatment.

The apparent 'difference' attributed to the Cendehill haemagglutinin may however be a function of the variation in response of all the strains to, for instance, high pH during alkaline extraction, or the effects of freeze thawing or the effects of passing on the virus to obtain higher titres of HA. It should be noted here, that these results were obtained with sera from single animals and may reflect specific factors of the particular animal influencing the results. Obviously too little information exists to be certain of any mechanism, and all that can be stated is that differences were noted in antisera produced by the Cendehill strain. Further work is necessary at every point, from production of immunizing and haemagglutinating antigens to antibody response in groups of rabbits.

In order to determine the optimum conditions for estimation of the K value of the neutralization reaction a model system of standard virus and antiserum was investigated. It was found that first order kinetics
were operable in the first 15-19 minutes of the neutralization curve of low dilutions of antiserum and virus. At high dilutions unexpected deviations from a typical neutralization curve were encountered after the initial first order reaction. Whatever factor caused these deviations a) was present in both immune and pre-immune serum from the Californian strain of rabbits, b) was only seen in higher dilutions of immune serum, c) was heat labile and could be removed by heating sera at 56°C for 30 minutes but could not be restored by the addition of fresh guinea pig serum at a dilution of 1/50.

The effects could not be explained by heterogeneity of the antigen population or inaccuracies of the assay system for they did not occur in all the reaction mixtures investigated. The effect was only noted as the antisera were diluted, possibly suggesting that as the antibody content relative to antigen lessened, unstable aggregates were formed which readily dissociated. Lafferty (1963) suggested that the neutralization reaction proceeded in two stages, the first in which dissociable antigen-antibody complexes were formed and the second in which these were stabilized. Thus, at lower levels of antibody unstable complexes might break down releasing virus, perhaps on dilution on the cell sheet during infection of the plates, however such a phenomenon would not account for the effects observed in pre-immune serum. Rawls et al (1967) reported that some batches of non-immune guinea-pig serum showed inhibition of rubella virus, and Almeida et al (1969) found that such sera caused aggregation of rubella virus particles; this factor however was heat stable. It may be that rabbit sera contain a similar factor which is heat labile, but which can cause unstable aggregation of the virus which breaks down yielding free virus, or which initially forms large rafts of virus which trap free virus within their structure rendering it non-infectious in the assay system. Such large rafts might be unstable and might eventually break down to
smaller units, possibly on dilution, releasing free virus.

In order to estimate the rates of the neutralization reaction, it was necessary to select conditions under which the phenomenon described above would not occur, thus lower dilutions of antibody were used, i.e. 1/8, this also satisfied the conditions of antibody excess necessary for the neutralization reaction to proceed, initially at least, by first order kinetics. (Andrews and Elford, 1933, Dulbecco et al, 1956). Heat inactivated serum would perhaps have been preferable, but it was felt that this might yield results which did not truly reflect the antibody present (Taylor-Robinson, 1969) and possible minor serological differences between strains might be missed. Addition of complement to such a system was known to enhance neutralization, however, it was noted that on addition of complement the period of first order kinetics was reduced to approximately 5 minutes and thus estimations of the slope of such a neutralization curve would have been based on a single reading, furthermore it was not certain that samples taken at five minutes would be on the part of the curve obeying first order kinetics.

Thus it was decided that unheated sera at low dilutions would be employed for estimations in homologous and heterologous systems to be taken over the first 10 minutes of reaction time.

Estimations of the K value for the standard control antiserum and control virus on thirteen separate occasions gave reproducible results with a minimum variation of 10 in the normalised values and a standard deviation of $\pm 7.093$. Insufficient data was available for further statistical analysis of these results and those of the homologous and heterologous antiserum/antigen mixtures. Estimations of K for the homologous systems within one test again gave a maximum variation
Cross neutralization tests in which the K values were estimated for a single serum against homologous and heterologous antigens yielded interesting results, with the normalised K values for the heterologous antigens being up to 70% lower than those obtained for the homologous system. Allowing that the maximum variation in repeated estimations of K values for any one system was found to be 10%, the results would seem to suggest the existence of some significant difference in the antigens used for antiserum production. The HPV-77 and Lesley strains were the only antigens encountered in which the K values were higher than those found for the homologous antigen with a particular antiserum. Fogel et al (1969) reported similar results with the HPV-77 strain in their simple cross neutralization tests and suggested that this might be an example of a strain particularly sensitive to antibody. The results obtained in this investigation would appear to support this view and it was noted that the lowpass Lesley strain exhibited the property to an even more pronounced degree than did the HPV-77 strain, particularly when employed for the estimation of antiserum to the HPV-77 and Dunning strains.

Similar phenomenon have been reported for Influenza A virus, (Choppin and Tamm, 1959, 1960), Echo virus type 6, (Karzon, Pollock and Barron, 1959) and the coxsackie B4 viruses (Choppin and Eggers, 1962). Karzon et al (1959), suggested a shift of Echovirus to a more 'antibody-sensitive' phase on passage in tissue culture with attendant changes in other properties such as increased virus yield and extent of cytopathic effect. Choppin et al (1962), however put forward the idea of a mixed population of virus with varied sensitivity to antibody, one type being selected under a given set of conditions and becoming predominant. Thus passage of the Echo virus type 6 selected the
with passage. It is possible that similar variations in sensitivity to antibody may exist between antigenically identical strains of rubella virus and that the HPV-77 and Lesley strains are examples of such phenomenon, furthermore it may be possible that the difference in passage history of these two strains accounts for the apparent variation in the extent of the sensitivity to antibody.
It has been possible to employ the existing plaque techniques available for rubella virus, in order to obtain a system which with minor modifications gave reliable and reproducible results on plaque morphology and infectivity assay. This technique allowed detailed comparisons of the plaque morphology of strains of rubella virus from varied sources to be undertaken, and the influence of tissue culture passage on this property to be studied. The availability of an accurate plaque assay system for infectivity allowed the finer comparisons of thermal stability and the kinetics of neutralization to be undertaken and added confidence to the comparative study of the growth characteristics of rubella strains in selected tissues.

It has been shown that the normal plaque size of low pass rubella strains lies on the small end of the scale in the region of 1.0mm, and these results would seem to support the evidence of low cytopathogenicity for GL-RK_{13} cells, put forward by such authors as Oxford (1969). It has not been possible to elucidate the influence of passage on plaque size and no clear relationship has emerged. It must be noted however, that only limited passage of selected strains was undertaken and very few of the strains investigated were obtained originally as fresh clinical specimens without previous laboratory manipulation. Similar findings were reported by Morgan (1969) who again was unable to show a direct relationship between plaque size and passage history. It would seem unlikely that passage through any selected tissue would always result in an increase in plaque size in GL-RK_{13}, for examination of the plaque morphology of the three extensively passaged vaccine strains, HPV-77 (Parkman et al, 1969), Cendehill, (Huygelen et al, 1969) and RA27/3, Plotkin, 1969) shows a range of plaque size from small to large. It would seem reasonable
to expect different strains to respond individually in their plaque morphology, the type of response, be it an increase, decrease or complete lack of change, being dependant on other factors also, such as the tissue selected or the previous history of the strain in question. Unless strains of rubella are isolated and handled in one laboratory and always passaged in parallel through single batches of tissue, it is not possible to rule out the influence of variation in a particular cell line from laboratory to laboratory or even variation with time in one cell line handled continuously. The apparently unique Dunning and Janine strains encountered in this investigation, with their large plaque type may be examples of this phenomenon. The variation in the history of such strains may even extend beyond the in vitro cultivation to the in vivo, uterine replication, for it is interesting to note that the large plaque type was not found in strains isolated from postnatal infections and the evidence relating the Janine strain to Dunning could not be ignored. Certainly, changes in property such as immunogenicity in rabbits have been shown to be altered by in vivo passage, (Oxford et al, 1970 and Gill et al, 1973), a phenomenon which would also account for the differences between Janine and Dunning strains. It is disappointing that a clear relationship between attenuation and plaque size has not emerged for it cannot be denied that such a marker would provide a valuable and easily monitored method of distinction.

Some variation in the growth characteristics of the strains examined in various tissue culture systems have also been demonstrated, but once again the influence of the previous history of such strains cannot be ruled out. This was clearly illustrated with the growth of the monkey tissue passaged HPV-77 strain in Vero cells.
However, the apparent adaptation of the RA27/3 strain to growth in BHK21/13S cells, and its unique ability to form plaques in this system, (Plotkin, 1969), cannot be explained by previous passage in this tissue. There remains only the possibility of growth in unrelated tissue, namely human diploid cultures, predisposing the strain to growth in BHK21/13S cells, and if this is accepted, then it is possible that similar phenomena exist with strains of rubella passaged in other tissues and may help to explain the differences in response to passage.

The differences in the thermal stability of the strains encountered in this study, may also be attributable to previous cultural history, furthermore it is interesting to note that the greatest distinction lay between the strains isolated from rubella syndrome infants, Dunning and Thomas, and those taken from postnatally acquired infections, Cendehill, HPV-77 and Lesley, or from foetal tissue taken early in the gestation period, RA27/3. This variation may be in part explained by a difference in the degree of aggregation of the virus pools, the most heavily aggregated being most resistant to the higher temperature due to the screening of the internal virions within the raft. Such properties as cytopathogenicity and aggregation may well be influenced by the conditions and tissue of culture, for a virus such as rubella which buds from cellular membranes such as the Golgi apparatus or the external surfaces of the cell (von Bonsdorff & Vaheri, 1969) will pick up cellular components which may well alter the virus properties phenotypically.

Striking differences in the immunogenicity in rabbits of the six strains of rubella were not encountered, however it was noted that after a single inoculation the titres of antibody produced to the Cendehill and RA27/3 strains were significantly lower than those induced by the remaining four strains. This apparent difference in immunogenicity
was overcome when repeated inoculations were administered, possibly indicating a lack of, or lower level of infection of the rabbits by the Cendehill and RA27/3 strains. Oxford (1969), Oxford et al (1970) and London et al (1971) have suggested that the attenuated strains of rubella induce lower levels of antibody in rabbits than do the virulent strains, however this was not supported by the findings reported here for the HPV-77 vaccine strain as compared to the low pass strains. It may be that the higher titres of inoculum used in this study may overcome the distinctions described in these reports, or possibly emphasize differences in the degree of attenuation between the vaccine strains. It is also likely that the previous treatment of the strain and route of inoculation have influenced the results reported here.

The Cendehill strain again appeared to differ in the cross haemagglutination inhibition studies; antibody seeming to fall off faster, or at least being detected at lower levels depending on the strain of haemagglutinin in use. Obviously, the results obtained must be in question as they are based on sera taken from a single animal, and Oxford (1969) and Gill et al (1973) similarly showed a variation in response between individual rabbits. However they do raise interesting questions to be considered in the detection and preparation of a strain of haemagglutinin for assay of antibody in serum samples. The fact that neutralizing antibody in Cendehill antiserum can be detected with RA27/3 virus but that the haemagglutination inhibiting antibody cannot, might possibly suggest that if a difference in the virion does exist, it may only involve a small part and not the intact virus which is involved in neutralization.

The estimations of the K value of neutralization were interesting in that two "antibody-avid" strains were found, HPV-77 and Lesley.
Fogel et al. (1969) had previously reported this phenomenon for the HPV-77 strain which was also shown to share the property of poor growth in BHK21/13S cells with the Lesley strain in this study. This avidity for antibody did not apparently extend to the haemagglutination inhibition reaction for the Lesley haemagglutinin was among those unable to detect Cendehill antibody in some rabbits' sera. The apparent lack of any very close relationship between the strains examined by kinetic neutralization may reflect an excessive variation in the test system, however, the reproducibility of values for the control antigen-serum mixture and the homologous mixtures would not appear to support this view. Where an obvious reaction of identity is seen, for instance, the Thomas antiserum against Cendehill and Dunning antigens it appears to be a one way cross only. A similar situation was encountered by McBride (1959) with poliovirus where each serum "uniquely identified" the homologous virus alone; he was, however, able to show some relationship between strains isolated in closely spaced outbreaks. This antigenic variation within virus type has not been shown to be of great import to the community for the relationships are sufficiently close for cross protection to occur. The situation would seem to be much the same for rubella virus, for no evidence has appeared in this investigation or in other reports which would suggest a strain variation sufficiently great to be of clinical importance.

It would seem possible that the differences in the properties of the strains reported here may be due entirely to variation induced by the previous history of these strains. Certainly many reports appear in the literature illustrating similar variation with some viruses, for example Finter (1964) reported a change in plaque
formation on L cells, interferon induction and sensitivity, and growth potential in calf kidney tissue for Semliki Forest virus after limited passage through calf kidney tissue, without a detectable antigenic change, Nicholson and Herrick (1969) demonstrated a more extensive change in Kilham Rat virus after passage in rat embryo cell cultures or suckling hamsters; after as little as three passages antigenic changes were evident in haemagglutination inhibition and neutralization tests, growth in the two systems differed and there was evidence to suggest differences in the components of the viral protein. Similarly variation has been shown to occur in vivo for Equine Infectious Anaemia virus (Kono, Kobayashi, and Fukuraga, 1973), with a continual drift in the surface antigens involved in the neutralization reaction occurring during the relapsing febrile disease of the infected horse.

If it is accepted that the minor differences between strains noted in this study are due to differences in the previous history of these isolates, and that passage in vitro or in vivo, be it the developing foetus, the postnatally infected individual or the vaccinee can elicit such variation in strains, the possibility arises that passage of a rubella virus through the population may alter some of its properties. Unexpected phenomena such as the lack of teratogenesis in Japanese strains of rubella, and the variation in severity of the infections in both postnatally and congenitally infected individuals may be accounted for in part at least by such factors.
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Appendix

A. Tissue Culture Media;

1. Eagles Minimal Essential Medium (M.E.M.)
   10 x concentrated (without sodium bicarbonate and antibiotics)
   Supplied by Wellcome Reagents Ltd.

2. Eagles Basal Medium (B.M.E.)
   10 x concentrated (without sodium bicarbonate and antibiotics)
   Supplied by Wellcome Reagents Ltd.

3. Eagles Medium (BHK)
   10 x concentrated (without sodium bicarbonate, antibiotics or
   tryptose phosphate broth)
   Supplied by Wellcome Reagents Ltd.

B. Tissue Culture Solutions;

1. Lactalbumin Hydrolysate (0.5%) in Earles Balanced Salt
   Solution (B.S.S.)
   10 x concentrated (without sodium bicarbonate and antibiotics)
   Supplied by Wellcome Reagents Ltd.

2. Liver Digest (Ultrafiltrate)
   Supplied by Wellcome Reagents Ltd.

3. Sodium Bicarbonate
   4.4% Solution
   Supplied by Wellcome Reagents Ltd.

4. Trypsin Solution
   5% (1:300)
   Supplied by Wellcome Reagents Ltd.

5. Tryptose Phosphate Broth
   Supplied by Wellcome Reagents Ltd.

6. Versene Solution (1:5000)
   Supplied by Wellcome Reagents Ltd.
7. Glucose Solution
   10% Solution made up with glucose (BR specification)
   Supplied by Brown and Polson.

8. Arginine
   1 Molar solution in distilled water
   Supplied by Wellcome Reagents Ltd.
   (Not on Sale)

9. Glycine
   1 Molar solution in distilled water
   Supplied by Wellcome Reagents Ltd.
   (Not on Sale)

10. Bovine Serum Albumin
    7% Solution made up with Bovine serum albumin
    Supplied by Armour Pharmaceuticals Ltd.

11. Adult Bovine Serum (Uninactivated)
    Collected from adult bovines on slaughter.
    (Not on Sale).

12. Calf serum
    a) Agamma calf serum; collected from colostrum-deprived
       calves and tested for Y globulin
       (Not on Sale)

    b) Fetal calf serum; collected on slaughter of pregnant bovines
       Supplied by Wellcome Reagents Ltd.

13. Difco Noble Agar
    Supplied by Difco Laboratories. Made up to 7% in distilled water
    and autoclaved. Reheated once only before use.
B. Diluents and Buffers.

1. Phosphate Buffered Saline
   Stored as 2 separate solutions; A and B+C.

   Solution A.
   Sodium chloride (AR) 10 gm.
   Potassium chloride (AR) 0.25 gm
   Potassium dihydrogen phosphate (AR) 0.25 gm
   Disodium hydrogen phosphate (AR) 1.4375 gm
   1% Phenol Red 1.0 ml
   Distilled Water to 1000 ml.

   Solution B+C.
   20% Calcium chloride solution 2.5 ml
   Magnesium chloride MgCl₂ 6 H₂O (AR) 0.5 gm
   Distilled Water to 1000 ml.
   Not on Sale.

2. Saline
   Made up at 0.85% in distilled water using NaCl (AR)
   Supplied by British Drug House.

3. Dextrose Gelatine Veronal buffer (D.G.V.) Buffer
   Supplied by Wellcome Reagents Ltd.

4. Glycine Buffer at pH 10. (approximately 2 Molar)
   40% Sodium hydroxide (AR) 127.5 ml
   Sodium chloride (AR) 104 gm
   Glycine 158 gm
   Distilled Water to 1000 ml
C. Miscellaneous components.

1. Crystal violet stain
Crystal violet powder (George T. Gurr, Searle Scientific Services), made up to a 10% solution in 70% alcohol.
8.0 ml of this solution added to 1000 ml of phosphate buffered saline containing 10% formaldehyde (Analar).
The stain is kept at room temperature and filtered immediately prior to use.

2. Neutral red stain.
Neutral red powder (George T. Gurr, Searle Scientific Services), made up to a 0.5% solution in distilled water.
Used at a final concentration of 1 in 20,000.

3. Heparin B.P. (Mucous)
Made up as a solution of 4,000 units/ml in distilled water.
Supplied by the Boots Company Ltd.

4. Manganese chloride (Analar)
Made up as a 1 Molar solution in distilled water
Supplied by BDH Chemicals Ltd.

5. Kaolin (acid-washed)
Supplied by Hopkin and Williams Ltd.

6. Rubella haemagglutinin
Edmund or Thomas strain, fluid + cell extract (alkaline buffer)
Tween-ether treated and freeze-dried.
Supplied by Wellcome Reagents Ltd.

7. Alsevers Solution (modified) Used in a 1:1 ratio with blood.
Dextrose 20.5 gm
Sodium Citrate 8.0 gm
Citric Acid 0.55 gm
Sodium chloride 4.20 gm
Distilled Water to 1000 ml
Sterilized by membrane filtration
Supplied by Wellcome Reagents Ltd.
D. Equipment.

1. Glassware.
   a) Soda glass
      1, 2, 4, 20 oz. and Universal bottles
      supplied by U.G.B. Ltd.
   b) Borosilicate glass
      Roux bottles and 6" x 5/8" test tubes.

2. Disposable Plastics.
   a) Falcon petri dishes
      Supplied in 35 or 60 mm sizes by Falcon Plastics
      (Division of Bioquest).
   b) Falcon microtitre tray + lid for tissue culture
      Supplied by Falcon Plastics.
   c) Microtitre trays (V-bottom) for serology.
      Supplied by Cooke Engineering Company (CECO)

   Supplied by Cooke Engineering Company (CECO)

4. L.T.E. Incubator
   Water jacketed, copper-lined model.
   Supplied by Laboratory Thermal Equipment Ltd.,
   a) Hyflo pump, Model B, supplied by Medcalf Bros.Ltd.
   b) pH Meters, Model 700, supplied by Analytical Measurement Ltd.
   c) Control valve, Type No. ACO2, supplied by Alexander Controls

   Dokamator DL/2
   Microfilm reader.
Abbreviations

AS. Antiserum
EMEM. Eagles Basal Medium
BSS. Eagles Balanced Salt Solution
CETC. Chick embryo tissue culture
CO₂ Carbon dioxide
cpe cytopathic effect
GM. Growth Medium
HA. Haemagglutinin
HAU. Haemagglutinating Unit
IND₅₀ Interfering dose₅₀
M. Molar
MEM. Eagles Minimal Essential Medium.
MK. Monkey kidney tissue culture
ml. millilitre
MM. Maintenance Medium
mm. millimetres
M. orale Mycoplasma
M. hominis 
P. passage level
pfu plaque forming unit
rpm revolutions per minute
sq.cm. square centimetre
TCD₅₀ tissue culture infecting dose₅₀
YRK. Young rabbit kidney.