BIOCHEMICAL AND CYTOCHEMICAL STUDIES
ON
RAT LIVER AND HEPATOMA

A Thesis submitted to the University
of Surrey for the degree of
Doctor of Philosophy

by

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SUMMARY

The main aims have been to separate membrane fragments as found in liver homogenates, and to develop and apply methods for cytochemical examination of the fractions obtained.

Fixation of tissue fractions in glutaraldehyde and incubation in lead-containing media resulted in a marked inhibition of enzyme activities in biochemical and cytochemical assays. Acceptable cytochemical results were obtained with unfixed fractions and also with sections prepared by a novel method. Incubation studies indicated no increase with time in the proportion of microsomal membrane fragments exhibiting 5'-nucleotidase or glucose-6-phosphatase activities, but a lag period was noted for glucose-6-phosphatase.

Zonal centrifugation of post-lysosomal fractions in a 'B-IV' rotor with a sucrose gradient gave three main peaks corresponding to soluble material, smooth-surfaced membrane fragments and rough-surfaced fragments. 5'-Nucleotidase (a plasma membrane marker) and glucose-6-phosphatase (a cytomembrane marker) were demonstrated biochemically and cytochemically in the smooth-surfaced fraction. Separation of 5'-nucleotidase and glucose-6-phosphatase was obtained in the presence of magnesium but not with caesium. No marked change in the position of the enzyme peaks resulted from the use of 0.12 M sucrose or 0.14 M NaCl in place of 0.25 M sucrose, or from different centrifugation times. Effective separation of 5'-nucleotidase and glucose-6-phosphatase was obtained by using sigmoid gradients. The resolution was not improved by the presence of deoxycholate. The distribution of ADPase and magnesium-activated ATPase closely paralleled 5'-nucleotidase, while IDPase and UDPase showed a distribution intermediate
between that of glucose-6-phosphatase and 5'-nucleotidase.

Fractionation of a crude nuclear preparation led to the demonstration of 5'-nucleotidase on sheets of plasma membrane and bile canaliculi, while glucose-6-phosphatase activity was detected in small vesicles adhering to the plasma membrane.

Cytochemical observations on hepatoma sections gave no evidence that the localizations of 5'-nucleotidase, glucose-6-phosphatase and ATPase within the 'parenchymal' cells differed from those found in normal liver. Zonal centrifugation of a 'post-lysosomal' fraction from a transplanted hepatoma showed a similar pattern to that of normal liver, as did a cytochemical study of hepatoma fractions.
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3. Zonal centrifugation of hepatoma 'microsomal material' to separate membranous elements.

Acknowledgments

References
(a) Abbreviations used in text.

<table>
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<tr>
<td>AMPase</td>
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<tr>
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<td>3'-METHYL-4-DIMETHYLAMINOAZOBENZENE</td>
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<tr>
<td>Hom.</td>
<td>Homogenate</td>
</tr>
<tr>
<td>15,000 g pellet</td>
<td>Pellet obtained by centrifuging homogenate at 15,000 g for 20 minutes.</td>
</tr>
<tr>
<td>Mic.</td>
<td>Microsomal pellet obtained by centrifuging 15,000 g supernatant at 55,000 g for 90 minutes.</td>
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<table>
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<tr>
<td>SH</td>
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<td>V</td>
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1. Introduction

The technique of differential centrifugation has frequently been applied as a means of isolating particles of biological interest, usually in the form of pellets in centrifuge tubes. Proper identification and characterisation of the elements in such pellets awaited cytological studies such as are considered later in this chapter.

For the separation of mixtures of particles whose sedimentation coefficients differ greatly, differential centrifugation is quite effective, despite the low resolution. However, in cases where very small differences in sedimentation rate exist it is of poor effectiveness. To overcome these difficulties and to achieve separations of higher resolution, two types of centrifugation have been developed. In the first type the particles are separated into discrete zones on the basis of differences in sedimentation rate. This is known as rate-zonal centrifugation, and the gradient serves merely to stabilize the zones. In the second type, known as isopycnic-zonal centrifugation, separation is based on differences in bouyant density (Anderson, 1966).

Although good separations have been achieved in density gradients set up in tubes, using swinging-bucket centrifuge rotors, many disadvantages are encountered. Great care must be taken so that the gradient is not disturbed during acceleration or deceleration. There are more disadvantages with high-speed rotors where wall effects may produce premature sedimentation of particles. The amount of material that may be separated using rate and isopycnic separations in swinging buckets is severely limited, the amount of tissue suspension that can be applied being under 1 ml per tube. With all of these disadvantages in mind, Anderson (1962) set about the task of developing zonal rotors with large capacities and high resolving power.
Use of such special rotors is considered later in this Chapter.

Zonal centrifugation in density gradients, in ordinary or special rotors, is a general method applicable to the separation of particles ranging in size from whole cells to small protein molecules. Behrens (1938), using non-aqueous gradients, employed isopycnic centrifugation methods for density measurements on biological components and also for separation purposes. Various authors have applied sucrose gradients, with or without the addition of other components, to the fractionation of virus or cell material (inter alia: Brakke, 1951, 1953; Holten, Ottesen, Weber, 1953; Schneider, Dalton, Kuff, Felix, 1953; Kuff and Schneider, 1954; Weber, 1955; Blaschko, Hagen, Hagen, 1957; Barnett, Hagen, Lee, 1958).

Gradient centrifugation is merely a special application of the centrifugal technique, complicated by the existence of gradients of density, viscosity and osmotic pressure within the tube. The results achieved by the conventional methods of differential centrifugation have been critically surveyed by de Duve and Berthet (1954); Hogeboom and Schneider (1955); Anderson (1956); Hogeboom, Kuff and Schneider (1957); and de Duve (1967). The theory and applications of gradient centrifugation have been reviewed by de Duve, Berthet and Beaufoy (1959).

In the present work, as in much published work, liver has been the main tissue employed. Liver is fairly homogeneous in cell type and for this reason it is frequently used for histological experimentation. It consists mainly of parenchymal cells but in fractionation studies the possibility of enzyme activities coming from non-parenchymal cells must be borne in mind. Fortunately glucose-6-phosphatase, an enzyme which hydrolyses glucose-6-phosphate to glucose and phosphoric acid, is known to be chiefly parenchymal (Wachstein and Meisel, 1956; Chang, 1960).
Membranes

Electron micrographs of the 'unit membrane' structure of various cells led several investigators to postulate that these membranes were constructed of layers of lipids and proteins. A triple-layer structure consisting of lipid sandwiched between two protein layers and which upon electron microscopic examination appears as two electron-opaque lines in close apposition, has been postulated (Danielli and Davson, 1935; Robertson, 1966). Cellular membranes (cf. Fig. 1), which are notable ultrastructural features of the animal cell, may be broadly classified as external (plasma) membranes, internal membranes (cytomembranes) and organelle membranes (Whittaker, 1968).

The outer surface of the plasma membrane is usually opposed to that of adjoining cells, and structural links (desmosomes), which represent modified stretches of the plasma membrane are reckoned to exist. In many cell types there are convolutions of the membrane, forming tubular ramifications (micro-villi) which comprise, in the case of hepatic cells, the bile canaliculi and the sinusoid wall.

Cytomembranes (endoplasmic reticulum membranes) are usually classified as rough-surfaced (granular) cytomembranes and smooth-surfaced (agranular) cytomembranes. The former are so-called because of the ribosomes that stud their surface and they are numerous in cells that synthesize protein for export. Both types of membrane form arrays of cisternae or tubules that intercommunicate with each other and possibly with the cell exterior. A specialized form of smooth membrane, characteristically observed as an isolated pattern of stacked cisternae, is known as the Golgi apparatus.
Membranes of subcellular organelles are the least homogeneous. The membranes comprising the nuclear envelope are believed to be homologous with the endoplasmic reticulum; they are studded with pores that may facilitate the exchange of macromolecules essential for the genetic control of the cell (Whittaker, 1968). The outer of the two mitochondrial membranes may also be homologous with the smooth endoplasmic reticulum (Parsons, Williams, Thompson, Wilson and Chance, 1967).

The literature on tissue fractionation contains several ambiguities with regard to the nomenclature for subcellular fractions. One example is the term 'microsomes' which was originally proposed as synonym for 'small granules' and had a purely operational significance. It served simply as an inclusive denomination for all subcellular entities requiring a relatively high centrifugal force for complete sedimentation. The term 'microsomal fraction' has been preferred to the term 'microsomes' since, as is pointed out below, structures seen electron-microscopically in the fraction do not exist in the same form in the cell itself.

3. Choice of homogenization and centrifugal techniques

In tissue fractionation the homogenization conditions employed play a major role in the distribution of subcellular components. Whittaker and Dowe (1965) studied the effect of homogenization conditions on subcellular distribution of synaptosomes in brain from rat and guinea pig. Using three types of homogenizer – Aldridge type, modified Dounce type, and Emanuel-Chaikoff type, Whittaker and Dowe studied their effect on the yield of synaptosomes as indicated by assays for acetylcholine. With the Aldridge type homogenizer, they found that the yield of the initial homogenate and crude mitochondrial fraction fell when the rate of shear increased. Using the Emanuel-Chaikoff type homogenizer, which depends
on a different principle, they found that the yield fell still further. By contrast the mild conditions of the modified Dounce type did not greatly affect the amount of acetylcholine in the initial homogenate, but the amount in the heavy tissue fragments was higher. Further, the yield of synaptosomes was lower than that obtained with the Aldridge type homogenizer using a moderate rate of shear. They mention that the choice of media for homogenization might influence the subcellular distribution.

Gradient centrifugation techniques have played a prominent role in the isolation of biological membranes. For the isolation of plasma membranes from rat liver, Emmelot, Benedetti and Rümke (1964a) used a modified form of the method described by Neville (1960). Emmelot's method consisted in homogenizing the liver in dilute bicarbonate, repeated differential centrifugation at low speed, followed by flotation in a discontinuous sucrose gradient. Because of gel formation this technique could not be used in the isolation of plasma membranes from hepatoma. However, the difficulty was overcome by homogenizing the hepatomas in dilute citric acid followed by centrifugation in bicarbonate and flotation in a discontinuous sucrose gradient. In this gradient the liver membranes were isolated at the interface of density 1.16 - 1.18, and the hepatoma membranes at the interface density 1.14 - 1.16. Hepatoma membranes contaminated by mitochondria were also isolated at the interface density 1.16. Pure membranes could not be obtained from hepatomas until the sucrose layer of density 1.14 was introduced. The sucrose density range used in the centrifugation was from 1.14 to 1.22. The morphological purity of the preparations was checked by phase contrast and electron-microscopy.

In contrast to the hypotonic conditions employed in the preparation of rat liver plasma membranes by Neville (1960) and Emmelot and Bos (1962),
Takeuchi and Terayama (1965) isolated a plasma membrane fraction using isotonic (0.25 M) sucrose containing 0.5 mM CaCl$_2$. In order to prevent fragmentation of the plasma membrane, perfused rat liver was gently homogenized by rotating the teflon pestle of a Dounce homogenizer by hand, rather than mechanically. A crude nuclear fraction was prepared by centrifuging a liver homogenate, which had been filtered through gauze to remove connective tissue, for 15 minutes at 2900 r.p.m. Repeated centrifugation on discontinuous sucrose gradients yielded a plasma membrane fraction at a density 1.16 - 1.20 interface. Examination of the fraction under the phase contrast microscope and the electron microscope revealed membranous sheets free from nuclei and mitochondria. Small vesicles, probably derived from the endoplasmic reticulum, were seen attached to plasma membrane profiles.

Takeuchi and Terayama (1965) investigated the chemical composition of their plasma membrane preparations and stated that plasma membranes were composed of; 47% protein, 29% phospholipid, 5.5% cholesterol and its ester, 0.1% sialic acid, 3.3% RNA and 1% hexosamine. The amino acid composition of proteins from their preparations showed no resemblance to collagen. However, unlike Neville (1960) and Emmelot et al. (1962, 1964), these authors did not carry out an enzymic examination of their plasma membrane preparations.

In order to study intracellular membranes the plasma membrane must first be disrupted. Usually gentle homogenization is carried out to ensure minimum damage to mitochondria, lysosomes and other organelles. The starting material for plasma membrane separation from liver has been either a crude nuclear fraction - this, in effect, being the material used
in Emmelot's laboratory – or a microsomal fraction. As will be documented later 5'-nucleotidase, an enzyme which specifically hydrolyses both purine and pyrimidine mononucleotides with the phosphate group attached to the fifth carbon atom of the pentose and yielding nucleosides and phosphoric acid (El-Aaser, 1965; Hardonk, 1968), is a suitable 'marker' for the plasma membrane. Under conventional conditions the recoveries of 5'-nucleotidase in the nuclear and microsomal fractions are approximately 40% and 60% respectively (de Lamirande, Allard and Cantero, 1958; Segal and Brenner, 1960; Novikoff and Heus, 1963; Reid, El-Aaser, Turner and Siebert, 1964; El-Aaser and Reid, 1965).

El-Aaser and Reid (1965) drew attention to the fact that the 5'-nucleotidase activity of microsomal fractions was not associated with the endoplasmic reticulum. Their conclusions were based partly on the biochemical finding of a difference in intracytoplasmic distribution between 5'-nucleotidase and glucose-6-phosphatase and also, less conclusively, on a difference in response to deoxycholate. Cytochemical observations (in agreement with Novikoff, 1964) showed that dephosphorylation of 5'-mononucleotides occurred mainly in the plasma membrane. The same authors put forward strong evidence that the Mg^{++}-dependent dephosphorylation of nucleoside-5'-monophosphates, as distinct from diphosphates (Novikoff, 1964), was due to a single enzyme.

The plasma membrane fragments isolated from a lysed nuclear fraction consist of large planar sheets and elements resembling bile canaliculi (Emmelot et al., 1964). El-Aaser, Fitzsimons, Hinton, Reid, Klucis and Alexander (1966a) isolated plasma membrane fragments from a hepatic nuclear fraction, using a zonal rotor (see above). Electron microscopic examination
of the plasma membrane fraction revealed large sheets. Some vesicular material, probably derived from the endoplasmic reticulum, was also present.

(a) Work with microsomal fractions

The distribution of plasma membrane fragments between the microsomal fraction and the nuclear fraction depends on the homogenization conditions employed. Vigorous conditions, e.g., disruption of cells with a nitrogen pressure homogenizer (Hinton, Klucis, El-Aaser, Fitzsimons, Alexander and Reid, 1967), results in severe fragmentation of the plasma membrane and may lead to an increase in its recovery in the microsomal fraction, although still only as a minor component. Palade and Siekevitz (1956) and Tashiro (1957) pointed out that during homogenization, fragmentation of cytomembranes (rough-surfaced, smooth-surfaced and Golgi) appears to occur and by a 'pinching off' process gives rise to vesicles. On a weight basis these vesicles are the main constituent of the microsomal fraction.

Sucrose solutions of varying molarities are the media most frequently used in the isolation of microsomal fractions. Isotonic sucrose (0.25 M) and 0.68 M sucrose are used - the latter insures a better morphological preservation of cell organelles than methods using either isotonic sucrose or saline (Palade and Siekevitz, 1956). As a compromise, 0.4 M sucrose has been used by some workers. Pioneers of differential centrifugation used media lacking sucrose but containing salts. Campbell, Colper and Hicks (1964); Tashiro and Ogura (1951); and Dallner (1963) made use of media containing sucrose and neutral buffer in trace amount, sometimes including KCl and MgCl$_2$. MgCl$_2$ served to minimize damage to ribosomes and polysomes. It has been pointed out by de Duve (1967) that sucrose solutions are not always superior to ionic media, as was originally
believed (de Duve and Berthet, 1954). Salts tended to promote agglutination in liver homogenates but the reverse was true with spleen preparations which actually showed more agglutination in 0.25 M sucrose than in 0.2 M KCl (Bowers, 1964).

For sedimentation of the microsomal fraction with 0.25 M sucrose the centrifugation used by many authors has a value of the order of 6,000,000 g-min. This unit represents the product of the time of centrifugation in minutes and the relative centrifugal force g. The fraction is normally obtained as a gelatinous reddish pellet, superimposed on a pellet of particulate glycogen if the animals were not fasted.

Wallach and Kamat (1964) and de Duve (1964) discussed the advantages of density-gradient centrifugation over differential centrifugation, especially if judicious conditions are chosen. Indeed, separation of different elements in the microsomal fraction is hardly possible by classical differential centrifugation (Reid, 1967). Dašner (1963) carried out studies on the structural and enzymic organization of the membranous elements of liver microsomes. Rat liver was homogenized in 0.25 M sucrose with a glass-teflon homogenizer and was centrifuged (10,000 g for 20 minutes) to remove cell debris, nuclei and mitochondria. Density gradient centrifugation of the '10,000 g supernatant' in the presence of Cs+ ions (15 mM) gave rise to a fluffy, double-layered fraction and a pellet which, upon electron microscopic examination, was seen to consist of rough-surfaced vesicles and free ribosomes. Further density-gradient centrifugation of the interface fraction in the presence of Mg++ ions (10 mM) yielded a pellet of Mg++-binding smooth-surfaced vesicles (i.e. membranous elements lacking ribosomes) and a fraction which when pelleted consisted of Mg++-free
smooth-surfaced vesicles. The rough and the Mg\textsuperscript{++}-binding smooth vesicles displayed a similar pattern of enzymes, this differing greatly from that observed in the Mg\textsuperscript{++}-free smooth vesicles. The two former fractions contained all the glucose-6-phosphatase and a large part of the nucleoside diphosphatase activity (UDP or IDP as substrate). The nucleotide triphosphatase activity (ATP or CTP as substrate) was evenly distributed in the three fractions. Chemical analyses demonstrated a relatively high RNA/protein ratio and a low phospholipid/protein ratio in the rough vesicles. The ratios were inverted in the smooth-vesicle fractions. Dallner concluded that the rough and the Mg\textsuperscript{++}-binding smooth vesicles originate from the rough-surfaced and smooth-surfaced areas of the endoplasmic reticulum, and that the Mg\textsuperscript{++}-free smooth vesicles are not related to the endoplasmic reticulum. Procedures similar to that used by Dallner have been used by many workers in studies of the enzymology of microsomal fractions (Campbell et al., 1964).

Carvalho, Sanui and Pace (1965) isolated a rat liver microsomal fraction by differential centrifugation, as described by Sanui and Pace (1959). The microsomal fraction was subfractionated in D\textsubscript{2}O-sucrose solutions of various densities. The first subfraction, which was isolated at a density greater than 1.23, had a relatively high nucleic acid content and a low lipid content (termed nucleoprotein subfraction). The subfraction isolated with a density ranging between 1.12 and 1.18 had a low nucleic acid content and a high lipid content (termed lipoprotein subfraction). Investigating the binding of Ca\textsuperscript{++} ions and Mg\textsuperscript{++} ions by the two subfractions, Carvalho et al. (1965) observed that the nucleoprotein fraction bound 0.35 - 0.40 meq. of Ca\textsuperscript{++} or Mg\textsuperscript{++} per g. dry weight of nucleoprotein, whereas the lipoprotein fraction bound only 0.12 meq. Ca\textsuperscript{++} or Mg\textsuperscript{++} per g. dry weight of lipoprotein. Nevertheless, the lipoprotein fraction of the microsomal fraction was responsible for 45% of the total binding of Ca\textsuperscript{++} or Mg\textsuperscript{++} by the whole microsomal fraction. The authors' observations supported the contention that cellular membrane structures can bind divalent cations.
The distribution of phosphatase enzymes amongst the various membranes isolated from a microsomal fraction has been extensively investigated. Some cytochemical observations will now be considered. A detailed cytochemical survey will be presented later.

Workers in the laboratory of A.B. Novikoff carried out a cytochemical examination of 60 cell types and concluded that in no cell can cytomembranes dephosphorylate nucleoside mono- or tri-phosphates or ADP or CDP (Goldfischer, Essner and Novikoff, 1964). The same authors concluded that glucose-6-phosphatase was intimately associated with cytomembranes, whereas UTPase or ATPase and ADPase were mainly in the plasma membrane, especially where it folds to form microvilli. They also concluded that the enzyme which can split GDP, IDP or UDP (but not ADP or CDP) was located in both cytomembranes and the plasma membrane.

Analyses for glucose-6-phosphatase, esterase, NADH diaphorase and Mg\(^{++}\)-activated ATPase revealed the microsomal smooth and rough endoplasmic reticulum fractions of normal liver to be similar in these enzyme properties (Khairallah, Murray, Meyer and Pitot, 1967). Hepatoma fractions exhibited less glucose-6-phosphatase and diaphorase activity than liver, but Mg\(^{++}\)-activated ATPase levels were elevated in tumour microsomal fractions. Observations on both liver and hepatoma emphasized the biochemical similarities between the smooth and rough endoplasmic reticulum.

(c) Membrane isolation with zonal rotors

As mentioned earlier, the lack of capacity of centrifuge tubes and their liability to 'wall effects' are among the disadvantages encountered with conventional centrifugal techniques. Work carried out during 1955-58 by
Anderson and his colleagues, led to the construction of a zonal rotor in which the operations of gradient formation, sample layering, particle separation and gradient recovery are all carried out during rotation in hollow rotors containing sector-shaped intercommunicating compartments. (See Chapter II for operational techniques with a "B-IV" zonal rotor.)

No tubes are required as in the case of conventional centrifugation, but only one sample can be handled. The large capacity (1.7 litres of gradient for the "B-IV" rotor) and good resolution renders zonal rotors valuable in the separation of particles in a size range extending from whole cells to protein molecules.

El-Aaser, Reid, Klucis, Alexander, Lett and Smith (1966) achieved partial separation of 5'-nucleotidase-containing elements from those containing glucose-6-phosphatase when rat liver microsomal material was centrifuged, using a "B-IV" zonal rotor. A better but still incomplete separation was achieved in the presence of 0.005 M MgCl₂. Further studies, concerning the improved separation of the components of microsomal material from rat liver are reported in this thesis.

4. Further literature on membrane isolation

In the isolation of toad bladder epithelial cell membranes, Hays (1966) used a modification of the method of Neville (1960). With a Dounce homogenizer, a cell suspension in bicarbonate solution was gently homogenized at 0° with 15 to 25 back-and-forth strokes of the pestle. The homogenate was freed from whole cells and nuclei by a method of filtration described by Warren (1966). The homogenate was collected and centrifuged for 30 min. (15,000 g at 4°). The membrane pellet was resuspended in bicarbonate solution and mixed with sucrose of density 1.34. This sucrose solution was transferred to a centrifuge tube, overlaid with sucrose of density 1.26 and finally with sucrose of density 1.16. Following centrifugation at 105,000 g for 75 min, a white band composed
of cell membranes was observed at the density 1.16 - 1.26 interface. The membranes were collected, suspended in bicarbonate solution and centrifuged, yielding a pellet of membranous material free from mitochondria and cytoplasmic debris as judged by phase contrast and electron microscopy.

Warren, Glick and Nass (1966a) isolated the surface membranes of animal cells by various methods. They found that animal cell surface membranes grown in tissue culture had no auxiliary support and were so delicate that it was very difficult to separate them in a reasonably intact state from cells. This difficulty was overcome by Kamat and Wallach (1965) who converted cellular membranes into vesicles by subjecting cells to a sudden decompression. Vesicles thus formed from plasma membranes, endoplasmic reticulum and other sources are then separated by isopycnic centrifugation. Warren's approach was to stabilize the surface structure so that it would withstand the rigours of separation and isolation from other cellular components. Strengthening of the membranes was brought about by lowering the pH to below 6, by using sulphydryl blocking reagents such as fluorescein mercuric acetate or 2-nitrobenzoic acid, or possibly by use of heavy metal ions. Tris buffer was alternatively used; it should yield membranes suitable for enzymatic studies since use of chemicals that might interfere is obviated. Eichholz and Krane (1965) used Tris buffers in the isolation of surface membranes of intestinal epithelial cells. Once stabilized, large fragments of surface membranes or whole-cell ghosts were isolated by centrifugation in solutions of sucrose or glycerol. Warren et al. (1966a) pointed out that their isolation procedures were successful when carried out on mouse fibroblasts, and that the surface membranes of other cells could probably be isolated by using essentially the same methods, but with minor changes in the forces and times of centrifugations.
Franke (1966) evolved a method for the isolation of nuclear envelope membranes from plant tissue. With the tips of adventitious roots of onion bulbs as starting material, nuclei were isolated in a combined differential and discontinuous gradient centrifugation following Kuehl's method (1964). Clumping of nuclei was avoided by omitting Ca\(^{++}\) ions, in agreement with Accola (1960). The pellet of nuclei was resuspended in a few drops of isolation medium to which distilled water or 0.02 M sucrose solution was added. This caused rapid swelling of the nuclei, rupture of the nuclear envelope, and subsequent formation of ghosts. The suspension of ghosts was then gently sonicated, layered over a 62% (w/v) sucrose solution and centrifuged for 30 min, at 3,000 \(\times\) g. The fraction at the density boundary containing the envelope membranes was then collected by conventional methods. Without giving specific details, Franke (1966) stated that the method for isolating nuclear envelope membranes from plant tissue could also be applied to animal tissues.

After commencement of the present study, Song and Bodansky (1966) published a method for the purification of 5'-nucleotidase from human liver. Their method contained many steps and the purpose was to eliminate nonspecific phosphatase activities; they did not first isolate a membrane-containing fraction. The purified fraction obtained had a ten-fold increase in specific activity of 5'-nucleotidase over the initial homogenate. In the absence of metal ions the pH optimum was in the range 7.1 to 7.5, while in the presence of 10 mM Mg\(^{++}\) a second pH optimum occurred in the region 9.3 to 9.5, probably due to the same enzyme.

Later, Song, Tandler and Bodansky (1967) prepared a membrane fraction rich in 5'-nucleotidase, from human liver. They claimed that 5'-nucleo-
tidase was solubilized by treatment with deoxycholate, a surface-active agent; and by a precipitation method employing ethanol, its activity was reassociated into membranous vesicles. The 5'-nucleotidase-containing vesicles were purified by centrifugation through a density-gradient solution of sucrose until the membranes reached positions of equal density. A sharp band of material with density corresponding to 1.06 was noted which contained about 70% of the total 5'-nucleotidase initially layered on the gradient.

5. Cytology as an aid to biochemical work

Most of the existing data on the intracellular localization of enzymes has been obtained by the biochemical approach, entailing determinations of enzymes in various cell fractions obtained by ultracentrifugation. These studies have yielded substantial information on the location of intracellular enzymes and have contributed greatly to the understanding of cell metabolism. However, there are many disadvantages which accompany such studies. One is the impurity of cell fractions. Another, already mentioned, is the existence of different cell types in a mammalian tissue such as liver. Studies on the morphology of isolated material can appropriately be considered here, with no attempt to cover the tissue (histology) on which such studies depend.

Novikoff (1956) pointed out that from the very inception of the techniques of differential centrifugation, biochemists have been concerned with the cytological characterization of the sedimented fractions. Using conventional light microscopes and phase-contrast microscopes, they were able to identify unbroken cells and organelles such as nuclei and mitochondria in homogenates from tissues such as liver. The resolution obtained with the light microscope was not sufficient to reveal small membrane
fragments. Vesicular material derived from plasma membranes or cyto-
membranes is visible only if the electron microscope is used. The
application of electron microscopy to isolated fractions is a relatively
recent development. It entailed handling centrifugal pellets by methods
already developed for intact tissue, with cutting of ultra-thin sections,

Littlefield, Keller, Gross and Zamecnik (1955) prepared cytoplasmic
ribonucleoprotein particles from the microsomal fraction of rat liver by use
of deoxycholate. The particles had a RNA to protein ratio approaching one
and included almost all the RNA of the microsomal fraction. In the
electron microscope, unfixed preparations showed essentially homogeneous
dense particles, which were approximately 240\AA\ in diameter. Microsomal
material which was fixed in osmium tetroxide, did not differ significantly
from unfixed material.

Palade and Siekevitz (1956) examined rat liver, liver homogenates
and microsomal fractions prepared from liver homogenates in the electron
microscope in sections of osmium tetroxide-fixed, methacrylate-embedded
tissues and pellets. They stated that most of the material examined
in the microsomal fraction had morphological resemblances to the rough
surfaced elements of the endoplasmic reticulum of hepatic cells. They
identified membrane-bound vesicles, tubules, and cisternae, which contained
an apparently homogeneous material of noticeable density, and studded with
dense particles (100 to 150\AA\) on their outer surface. In addition to the
rough surfaced membranes, the microsomal fractions contained a small
number of vesicles, free of attached particles and probably derived from
the smooth surfaced regions of the endoplasmic reticulum or the plasma
membrane,
When a microsomal fraction was treated with deoxycholate and centrifuged, it yielded a small pellet, which revealed dense particles (100 to 150Å) with only occasionally interspersed vesicles, when examined under the electron microscope. The different types of membranous elements observed by Palade and Siekevitz (1956) led them to conclude that the microsomal fraction was not homogeneous from a cytological standpoint.

In confirming and extending the work of Palade and Siekevitz (1956), Moule, Rouiller and Chauveau (1960) isolated a microsomal fraction from rat liver and further treated it to obtain two subfractions as pellets. The first fraction (centrifugation for 2 hours at 40,000 g) consisted of rough-surfaced vesicles while the second fraction (centrifugation of the supernatant at 145,000 g for 3 hours) consisted of smooth-surfaced vesicles, free particles and ferritin, when examined under the electron microscope.

In the isolation of subcellular fractions the amount of cross-contamination is often quite considerable. Emmelot et al. (1964b) isolated plasma membranes from rat liver and hepatoma and checked contamination with mitochondria and other cell organelles by phase and electron microscopy. They stated that the preparations, when examined under the electron microscope, were not contaminated by other identifiable cellular components such as nuclei, mitochondria, dense bodies, lysosomes and rough-surfaced cytomembranes. However, the possibility of contamination by smooth-surfaced cytomembranes must be borne in mind.

Schnaitman, Erwin and Greenawalt (1967) observed that controlled osmotic lysis of rat liver mitochondria resulted in a mixed population of small vesicles derived mainly from the outer mitochondrial membrane,
and of larger bodies which contained a few cristae and were derived from the inner membrane. The vesicles were separated from the larger bodies on Ficoll and sucrose gradients and examined under the electron microscope. Biochemical assays indicated that the small vesicles were rich in monoamine oxidase, and it was concluded that this enzyme was found only in the outer mitochondrial membrane.

The electron microscopic examination of subcellular fractions has often been performed on material collected by classical methods of centrifugation. Particles thus obtained may be present in a single pellet, yet are heterogeneous, in sedimentation coefficient or in density, with corresponding differences in morphology. The distribution of the different types in the pellet is not uniform and it is necessary to cut at various levels. Baudhuin, Evrard and Berthet (1967) described a method for preparing very thin pellicles of packed particles, by filtration on millipore filters. These pellicles could be embedded for electron microscopic examination of morphology and also of cytochemical characteristics. The main advantages of this method over the usual centrifugal packing techniques were that the heterogeneity was solely in the direction perpendicular to the surface of the pellicle and that a section covering the whole depth of the pellicle could be photographed in a single field.

Cytochemical as distinct from morphological observations will now be considered. Enzyme histochemistry at the light microscope level is of value mainly for ascertaining which type of cell in a heterogeneous tissue contains particular enzymes (Reid, 1967). However, electron microscopy has offered the possibility of escaping from limitations of the light microscope and of transforming enzyme histochemistry into a more precise tool. Some points of the technique will now be considered.
In the present context the term 'cytochemistry' incorporates enzyme localization studies carried out at both the light microscope and the electron microscope level.

Quantitative information concerning the intracellular location of enzymes would be greatly enhanced if a cytochemical assay were available for each enzyme investigated (Reid, 1967). The general procedure in cytochemical work is to incubate a tissue sample, already lightly fixed, in the presence of the substrate and of a 'product-capturing' agent, which in the case of phosphatase localization studies is usually Pb++ or Ca++ ions. The tissue sample, after further fixation is embedded and thin sections examined. Tissue blocks were used for incubation in early cytochemical work with the electron microscope (Persijn, Daems, de Man and Meijer, 1961). However the penetration of fixatives or substrates in tissue blocks is reckoned to be slower than in the tissue sections which are now preferred (Essner, Novikoff and Masek, 1958; Holt and Hicks, 1961; Sabatini, Benach and Barneett, 1963; Goldfischer, Essner and Novikoff, 1964).

In cytochemistry, one serious source of unreliability is the possible diffusion of the reaction product. Such diffusion could account for the observation that nuclear staining for 5'-nucleotidase is sometimes demonstrable, if the concentration of Pb++ ions in the incubation medium is rather low (Novikoff, Hausman and Podber, 1958; Reid et al., 1964; El-Aasser, 1965) or if the incubation time is prolonged (Goldfischer et al., 1964). The possibility of diffusion of reaction product in unfixed tissue will be dealt with in the Discussion.
(a) **Fixation**

The techniques of enzyme histochemistry which led, with little modification, to the present techniques of electron cytochemistry are still subject to rigorous criticism on the grounds of false localizations. In the preparation of tissues for enzyme histochemistry by light microscopy there are a number of important considerations, each of which is equally applicable in the case of electron microscopy. The first and probably the most important is the question of fixation. Pearse (1963) mentions three prime requisites for successful fixation of enzymes: (1) the preservation of enzyme activity, (2) the maintenance of the true localization, and (3) the preservation of the morphological structure of the cell. In the localization of acid phosphatase, Essner and Novikoff (1960, 1961), and Holt and Hicks (1961) found that the activity of the enzyme was reduced by fixation to about 50% as judged by assays on tissue blocks. Essner, Novikoff and Masek (1958) found that fixation with osmium tetroxide (OsO$_4$) leads to extensive loss of both ATPase and 5'-nucleotidase, assayed biochemically, and that the ATPase activity of mitochondria was completely eliminated. Although brief fixation in OsO$_4$ is used by some workers, the fixatives commonly used in enzyme cytochemistry are aldehydes.

Sabatini, Bensch and Barnett (1963) reviewed the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. Of seven aldehydes studied, glutaraldehyde gave the best general preservation of cellular fine structure. In the cytoplasm of the pancreatic acinar cells the most striking finding with glutaraldehyde-fixed material was the excellent preservation of the endoplasmic reticulum. When tissue already fixed in one of the aldehydes was briefly post-fixed in osmium tetroxide, the quality of preservation
was found to be comparable, and in some cases (e.g. glutaraldehyde and acrolein) equivalent to that obtained with the usual osmium tetroxide procedure.

Janigan (1964) studied the effects of aldehyde fixation on the enzymes β-Glucuronidase, β-Galactosidase, N-Acetyl-β-Glucosaminidase and β-Glucosidase in tissue blocks. In agreement with Holt, Hobbiger and Parvan (1960) he found that washing in gum sucrose after fixation, — served to remove excess fixative from the tissues. This permitted much higher recovery of enzyme activities than if it was omitted. Phosphate-buffered sucrose produced the same effect. Janigan (1964) observed high recoveries of enzyme activities after fixation in pyruvic aldehyde, acetaldehyde, hydroxyadipaldehyde or glyoxal. However with some of these fixatives the tissues were soft, with poor morphological preservation, and the enzyme activity that survived was found largely in a soluble and non-sedimentable form in water homogenates. The converse was found for tissues fixed in formaldehyde, glutaraldehyde, crotonaldehyde and acrolein.

In studying the interaction of aldehydes with collagen, Bowes and Cater (1966) found that with glutaraldehyde there was stabilization of collagen, due mainly to reaction with the amino groups. With glutaraldehyde the reaction was favoured by a rise in pH, temperature and concentration of aldehyde. The most important factor was pH, the optimum for reaction being pH 8.0.

A biochemical and histochemical comparison of the effects of formaldehyde and glutaraldehyde fixation on various enzymes was carried out by Hopwood (1967a). Increasing the incubation time with glutaraldehyde from 2 to 18 hours caused severe inhibition of the enzymes acid
phosphatase, catalase and β-glucuronidase. Hopwood (1967b)
fractionated glutaraldehydes on Sephadek G-10 in the pH range 3.0
to 8.2. Two fractions with absorptions at 235 \( \mu \)m and 280 \( \mu \)m were
separated, the latter alone having the aldehyde function. When
 glutaraldehyde was heated there was an increase in OD 280 which
was exponential in nature. There was also an increase in OD 280
with increasing pH above 4.6. The author suggested that the active
form of glutaraldehyde for fixation was the enol form, which
predominates at high pH values.

Anderson (1967) found that effective purification of commercial
 glutaraldehyde was achieved by distillation or by repeated washing
with charcoal of high surface area. Pure glutaraldehyde exhibited an
absorption maximum at 280 \( \mu \)m. A second maximum at 235 \( \mu \)m indicated
the presence of impurities which were probably of polymeric origin.
Representative oxidoreductase, transferase and hydrolase activities
were measured in supernatants and sediments of skeletal muscle
homogenates that had been treated with glutaraldehyde samples of low,
intermediate and high purity content, as judged by relative absorbance
at 235 \( \mu \)m. Anderson found that enzyme recovery was inversely related
to impurity content, the highest levels being obtained from tissue
treated with the purest glutaraldehyde.

In studying the inhibition of acid phosphatase by glutaraldehyde,
both commercial and distilled, Fahimi and Droschmans (1968) found
that the acidic products formed by oxidative decomposition did not
increase the inhibition. They suggested that the inhibition might
be due to the presence of high concentrations of inorganic phosphates
in different lots of commercial glutaraldehyde. Distillation of
 glutaraldehyde, which lowers its impurity content (Anderson, 1967)
resulted in a 10-20% decrease in the inhibition of acid phosphatase.

Further work on fixatives is considered in Section 6 (c) below, in connection with the electron microscopic findings.

(b) Effects of lead in relation to enzyme cytochemistry

As already mentioned, the divalent Pb^{++} serves to 'capture' the liberated phosphate in cytochemical studies involving phosphatase enzymes. However lead ions are known to strongly inhibit enzyme activities. Novikoff et al. (1958) stated that the low level of ATPase activity, assayed biochemically, in the Wachstein-Meisel medium was due to inhibition of enzyme activity by lead ions. Lowering the concentration of lead and increasing the concentration of ATP in the medium resulted in an increase of the enzyme activity. Unfortunately, no advantage could be taken of the increased enzyme activity in the staining procedure, since a high lead concentration was required for trapping the phosphate released during incubation of the sections. Lead ions also inhibit 5'-nucleotidase activity when assayed biochemically (El-Aaser, 1965).

Since commencement of this study, Rosenthal, Moses, Beaver and Schuffman (1966), Moses, Rosenthal, Beaver and Schuffman (1966) and Moses and Rosenthal (1967) reported the non-enzymic hydrolysis of ATP and other nucleoside phosphates by lead ions, in the Wachstein-Meisel medium. However, Novikoff (1966) repeated and extended some of the experiments of Rosenthal and Moses (cf. above) and concluded that the staining reactions obtained in the Wachstein-Meisel medium and similar media reflected real enzymic activities rather than non-enzymic hydrolysis.
(2) **Enzyme Cytochemistry**

In the following survey, phosphatases are given special attention. Cytochemical observations on various tissues, with especial emphasis on liver, are considered.

**Light microscopy**

Gomori (1939) and Takematsu (1939) independently developed a method for the light-microscopic localization of alkaline phosphate with a 'calcium phosphate' technique. From this method there arose a spectrum of methods for phosphatases active in the alkaline range, and also 'lead phosphate' methods for enzymes active at acid pH levels.

5'-Nucleotidase, which has a slightly alkaline pH optimum, was first demonstrated histochemically in testicular tissue by Gomori (1949), using a calcium phosphate technique.

Using 5'-AMP as substrate, Turchini and Legarde (1961) demonstrated 5'-nucleotidase activity in the sinusoids, bile canaliculi and periportal zones of mouse liver. Enzyme activity also seemed to be located in the parenchymal nuclei when IMP, which was dephosphorylated less rapidly than AMP, was used as substrate. Apparent localization of 5'-nucleotidase in nuclei, as well as in other sites, had previously been found by Wachstein and Meisel (1952) with acetone-fixed paraffin sections of different tissues.

By a lead phosphate technique, Wachstein (1955) demonstrated 5'-nucleotidase in human kidney using fresh frozen sections. Non-specific deposits are fewer in sections than in blocks, and the former should be preferred in spite of some damage to cellular structures.
Wachstein and Meisel (1957) studied 5'-nucleotidase activity in the liver of four species - rat, mouse, guinea-pig, and man. In fresh frozen sections high activity was found in the sinusoids. Better structural detail was preserved when sections were lightly fixed in formalin. Within the periportal fields connective tissue reacted positively in rat and guinea-pig.

5'-Nucleotidase was demonstrated in the secretory capillaries of the pancreas by Wachstein and Meisel (1959). The similarity between the liver and pancreas with regard to staining of canaliculi, located between the secretory cells, led the authors to suggest that the mechanism of transport between secretory cells and excretory channels is similar in the two tissues.

**Electron microscopy**

Electron microscopic techniques were later applied in the study of ATPase and 5'-nucleotidase of rat liver cells (Essner, Novikoff, and Masek, 1958). The fixative used was osmium tetroxide, which penetrates tissues so slowly that only thin sections from the periphery of tissue blocks could be used. The authors pointed out that the use of tissue blocks rather than sections restricts the penetration of substrate or other agents into the tissue. Exposure of tissue blocks to osmium tetroxide, even for a short period of time, resulted in a marked decrease in the activity of ATPase, and to a lesser extent, of 5'-nucleotidase, when assayed biochemically. Enzyme activity was observed in the plasma membrane of parenchymal cells, being greatest where the plasma membrane folds to form the microvilli of the bile canaliculus, when thin sections were incubated for ATPase or 5'-nucleotidase. 5'-Nucleotidase was also
localized in the microvilli of the sinusoids. A light deposition of lead phosphate on the nuclei of parenchymal cells was observed. The staining pattern observed for 5'-nucleotidase correlates well with the appearance in the light microscope of frozen unfixed sections or paraffin sections of acetone-fixed tissue (Essner et al., 1958).

For the fine structural localization of ATPase in the small intestine, kidney and liver of the rat, Ashworth, Louibel and Stewart (1963) concluded that the best results were obtained from tissue that had been quenched in liquid nitrogen and sectioned at 25μ in a cryostat. The tissue was then fixed for 30 to 90 minutes at 4°C in formalin-sucrose buffered to pH 7.2. It was incubated with substrate, and post-fixed with osmium tetroxide. Thin sections, cut from methacrylate embedded tissue, were examined under the electron microscope. Frozen sections of tissue, quenched in liquid nitrogen, fixed in formalin-sucrose and incubated with substrate, gave a better distribution of the ATPase localization product when examined in the electron microscope than did block-fixed and block-incubated tissue. As previously mentioned, this may be due to the slower penetration of fixatives or substrates into tissue blocks as compared with sections (Holt and Hicks, 1961).

In the context of fixation, Barrnett and Palade (1958) reviewed the applications of histochemistry to electron microscopy and suggested that a compromise should be made between their distinct requirements. Many enzymes, especially the dehydrogenases, are destroyed by any type of fixative, but some of the hydrolase enzymes are more rugged and will partially survive fixation, even in osmium tetroxide, if fixed for only a short time and at low
temperatures. The authors presented some work on the demonstration of dehydrogenase systems using potassium tellurite, which as a hydrogen acceptor is reduced by living tissues to an insoluble black product of high electron scattering power. They were able to localize succinic dehydrogenase activity in the mitochondria of cardiac muscle. Other work in the oxidoreductase field has been reported by Scarpelli, Hess and Pearse (1958), who studied mitochondrial diaphorase by a tetrazolium procedure.

Sabatini et al. (1963) reviewed the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. They concluded that excellent morphological preservation by fixation implied the suppression of enzyme activities. With regard to the choice of fixative, a compromise could be attained between a perfect general fixation with no enzymic activity and the retention of some activity in relation to a recognizable fine structure. In thick frozen sections of liver, which for phosphatases gave fewer nonspecific deposits of reaction product than in blocks, they demonstrated esterase activity, using thiolacetic acid as substrate, in the cisternae of the endoplasmic reticulum. Acid phosphatase activity in the liver and kidney occurred in many, but not all, of the dense bodies of the epithelial cells. The large dense bodies of macrophages in connective tissue and of Kupffer cells were constantly positive. ATPase activity was demonstrated in bile canaliculi and in sinusoids. In addition, peribiliary dense bodies were also reactive, probably owing to the activity of acid phosphatase. Plasma membranes and to a lesser extent bile canaliculi stained for 5'-nucleotidase, with AMP as substrate.

Pronounced enzyme activity was observed in the Golgi sacs and vesicles when IDP was used as substrate, and activity was also
demonstrated in the dense bodies with AMP and IDP as substrates.

Glucose-6-phosphatase activity which was best preserved with hydroxyadipaldehyde-fixed liver, appeared localized in the rough-surfaced endoplasmic reticulum. Unfortunately the glycogen areas were not well preserved in hydroxyadipaldehyde-fixed material, which made it difficult to establish the relationship between glucose-6-phosphatase activity and the membranes of the smooth-surfaced endoplasmic reticulum.

Orrenius and Ericsson (1966) examined the relationship of liver glucose-6-phosphatase to the proliferation of endoplasmic reticulum in phenobarbital induction. In hepatic parenchymal cells enzyme activity was demonstrated cytochemically in all endoplasmic reticulum membranes, including the phenobarbital-induced smooth-surfaced proliferates, even though there was an over-all decrease in activity.

Ashworth et al. (1963) demonstrated ATPase activity in the plasma membrane, bile canaliculus and sinusoids of rat liver. Lysosomes, lipid droplets and nuclei also exhibited some activity. In some experiments it was possible to demonstrate deposition of the reaction product in the cristae and outer membranes of mitochondria of the parenchymal liver cells. Due to the marked inhibition caused by fixation, other authors have failed to detect ATPase in mitochondria (Novikoff, Podber, Ryan and Noe, 1953; Novikoff, Hausman and Podber, 1958; Essner, Novikoff and Masek, 1958).

(d) **Tissue fraction cytochemistry**

It is only in recent years that the electron microscope has been applied to the enzymic study of tissue fractions.
Novikoff, Hausman and Podber (1958) studied the localization of ATPase in liver sections and in tissue fractions at the light microscope level. ATPase activity was greatly diminished when assayed biochemically in the medium of Padykula and Herman (1955), which contains Ca\(^{++}\) ions as a capturing agent, and is adjusted to pH 9.4. (Cysteine or other sulphydryl compounds were added to inhibit non-specific alkaline phosphatase while stimulating ATPase activity.) The addition of Mg\(^{++}\) ions had no effect upon the activity. ATPase activity in the medium of Wachstein and Meisel (1957), which is buffered at pH 7.4, contains Mg\(^{++}\) ions and utilizes Pb\(^{++}\) ions as a capturing agent, was also diminished; however this medium gave more consistent and more striking staining and was preferred to that of Padykula and Herman.

Formal-calcium fixation of tissues, as in the method of Novikoff et al. (1953), resulted in a loss of ATPase activity; the loss being much greater for mitochondria than for the nuclear fraction. It was suggested that the bile canaliculi present in the nuclear fraction are more rigid, more resistant structures than mitochondria, although the possibility of different enzymes being involved was not excluded.

When the concentration of lead ions in the Wachstein-Meisel medium was reduced, ATPase activity increased. However, this resulted in a decrease in staining intensity and led to artefactual staining of nuclei.

When frozen sections of rat liver were fixed with formal-calcium and stained by the method of Wachstein and Meisel (1957) for ATPase, enzyme activity was demonstrated in the portal triads and in bile canaliculi. Similar localization was found for 5'-nucleotidase.
A 10% suspension of a crude nuclear fraction in 0.88 M sucrose was incubated in a Wachstein-Meisel-type medium for ATPase and 5'-nucleotidase. The incubation for 5'-nucleotidase was conducted at an alkaline pH and utilized Ca^{++} ions to precipitate the enzymatically liberated phosphate. Following incubation, the mixture was centrifuged, washed, and suspended in sucrose. A drop of suspension was placed on a glass slide, inverted over a dish of ammonium sulphide and examined under the light microscope. Intense staining for ATPase and 5'-nucleotidase was observed in the bile canaliculi. Moderate staining of the contaminating cytoplasmic material was also demonstrated, but no staining in nuclei was observed.

The paucity of the literature in the field of tissue fraction-cytochemistry may be due to the difficulty of handling tissue fractions for enzyme cytochemical studies at the electron microscope level.

El-Aaser (1965) cytochemically examined sections of nuclear and microsomal pellets (cut on a freezing microtome) which had been fixed in 3% glutaraldehyde. In sections from a crude nuclear fraction, 5'-nucleotidase activity was demonstrated in fragments of membrane which "seemed to represent broken microvilli of bile canaliculi and blood sinusoids". No reaction could be detected in nuclei. Glucose-6-phosphatase activity was demonstrated in the nuclear envelope and in cytomembrane fragments. Electron microscopic examination of the microsomal fraction revealed 5'-nucleotidase staining in small rod-like membrane fragments, whereas glucose-6-phosphatase showed staining on vesicles derived from the endoplasmic reticulum. This pilot work, in which there may have been artefacts, has now been extended.
Some cytochemical literature on fractions has appeared since the present work was started:— the demonstration of Mg$^{++}$-activated ATPase associated with a microsomal fraction of rabbit psoas muscle (Tice and Engel, 1966); the localization of ATPase 'Mg$^{++}$ and Na$^+\text{-K}^+$-activated) on red cell ghost membranes (Marchesi and Palade, 1967); and the demonstration of acid phosphatase activity in a lysosomally-rich fraction isolated from rat liver (Baudhuin, Evrard and Berthet, 1967).

Cytology and Cytochemistry in relation to hepatocarcinogenesis

Hepatic tumours can be induced in several species of animals by a number of different cancer-producing agents, termed carcinogens, which are usually fed in the diet. Intensive histopathological studies of experimental liver cancer of the rat were initiated by the work of Yoshida (1932), who first reported the production of carcinomas of the liver by feeding the chemical o-aminooazotoluene in the diet. Since then, many chemical hepatocarcinogens have been discovered, e.g. dimethyl-aminoazobenzene and its derivations (Miller and Miller, 1953), ethionine and others.

Tumours usually take many weeks to appear after exposure of an animal to a carcinogen, and cannot be distinguished from one another on the basis of the agent used (Firminger, 1955). Rat hepatic tumours, loosely termed hepatomas, can be morphologically classified into either liver-cell type or bile-duct type on the basis of the general histological appearance of the cell mass.

Farber (1956) demonstrated that the early morphological alterations produced by many carcinogens, including ethionine, involve primarily an early proliferation of ductular cells from the portal triads of the liver. However, such observations on tissues examined after the first weeks of
carcinogen exposure ('precancerous' tissue) must be interpreted with caution as many of the changes are not specific for the ensuing hepatoma. Many workers were impressed by necrosis (apparent cell death) and regeneration as possible precursors in the formation of hepatomas. However, the relative lack of necrosis observed in the work of Firminger (1955) indicated that it was not necessarily a precursor.

To overcome the inconveniences involved in producing primary tumours, most biochemical research has been carried out with transplants. In the first instance primary tumour cells are inoculated into a normal animal, and the resulting transplants are maintained through successive generations by repeated transfers. Many primary and transplanted tumours of the rat were investigated in the hope of finding one closely similar to liver, to simplify the task of finding 'key' changes. The Morris hepatoma 5123, a slow-growing tumour of hepatic origin (Morris, 1963), exhibited morphological and biochemical properties which were very similar to those of normal and host liver. For this reason it was classified as a minimal deviation hepatoma (Morris, 1963).

Novikoff (1960) compared the Morris hepatoma 5123 with two rapidly growing tumours - the Dunning LC 18 hepatoma and the solid form of the Novikoff hepatoma. In biochemical respects the Novikoff hepatoma consistently differed from adult liver or from rapidly growing 'regenerating' liver induced by partial hepatectomy. The Dunning hepatoma bore more resemblance to the Novikoff than to the Morris hepatoma. Cytochemical observations on these hepatomas are considered later.
Biochemical studies of liver of carcinogen-fed rats are complicated by the fact that the investigator is dealing with a heterogeneous cell population that is changing as carcinogenesis proceeds (Pitot, 1962). Moreover, evidence has been presented that not only primary but also transplanted neoplasms are heterogeneous in morphology and in their enzymic properties. At the level of the light microscope, differences were observed between a primary hepatoma induced by ethionine and successive transplants from the same tumour (Pitot, 1962).

(a) **Ultrastructural studies**

"Membrane systems warrant continued study as possible primary sites for the actions of carcinogens and toxins" (Reid, 1967). Morphological lesions are detected, in the first instance, in the cytomembranes of liver cells treated with hepatocarcinogens such as azo dyes, or dimethylnitrosamine and with hepatotoxins such as carbon tetrachloride (Haguenau, 1958; Rouiller, 1964).

The finding that cancer could be transmitted through a single cell (Furth and Kahn, 1937) pointed to a mechanism of malignancy located at the cellular level and hinted that high resolution microscopy might reveal specific lesions. Bernhard (1958) carried out an electron microscopic examination of tumour cells and observed, as light microscopy studies had previously indicated, that cancer cells did not exhibit any morphological features which could be exclusively demonstrated in tumours as opposed to non-cancerous tissues. Nevertheless, certain tendencies which are characteristic of cancer cells in general have been recognised. One such tendency is dedifferentiation or anaplasia, and this loss of structural organization is often encountered at the electron microscope level. Bernhard (1958) observed less mitochondria, which were smaller in
size in tumour cells as compared with normal cells. Nuclei were larger in tumour cells and the endoplasmic reticulum membranes lacked an organized lamellar pattern. The plasma membranes were similar in appearance to those of normal tissues, this being in contrast to the observations of Emmelot et al. (1964a) who stated that with rat hepatoma, cell contact was poor.

Upon electron microscopic examination of the Morris hepatoma 5123, Novikoff (1960) found that tumour cells were similar in general appearance to hepatic parenchymal cells. He also observed that mitochondrial and microsomal fractions obtained by conventional centrifugation of tumour homogenates were cytologically more heterogeneous than those from liver, this being due to the smaller size of the mitochondria and the relative abundance of lipid spheres, Golgi membranes and plasma membranes in tumour cells.

In contrast to hepatomas of the minimal deviation type, the more anaplastic tumours show signs of ultrastructural disorganization (Mercer, 1961). Derangements of the endoplasmic reticulum are observed; the mitochondria appear to be more rounded; there are fewer rough-surfaced cytOMEMBRANES and more free risosomes. Many tumours, particularly on transplantation in animals, become progressively more anaplastic and this condition is characterized not only by a loss of internal organization but also by a progressive deterioration of intercellular adhesion.

Since commencement of this study many investigations on the fine structure of liver tumours induced in the rat by chemical carcinogens have been reported. Ma and Webber (1966) described two sub-types of a trabecular carcinoma induced in the rat by 3'-methyl-4-dimethyl-aminoazobenzene (3'-me-DAB). The first type consisted of polygonal
cells closely resembling normal liver cells, which formed bile canaliculi and possessed microbodies. The second type were distinguished by their great variation in cell size and shape, nuclear changes, grossly dilated and disorganized endoplasmic reticulum, and by the absence of both bile canaliculi and microbodies.

(b) Cytochemical studies

This section deals mainly with cytochemical studies carried out at the light microscope level.

With liver from rats treated with either carbon tetrachloride or thioacetamide (only the latter being carcinogenic in the rat), Wachstein and Meisel (1958) were unable to demonstrate 5'-nucleotidase in bile canaliculi of necrotic cells in the centro-lobular areas when normal conditions of incubation were used. However, prolonged incubation resulted in heavy staining of the central areas. The staining reaction was diffuse but seemed to be mostly localized in the surviving sinusoids and to a lesser extent in necrotic cells. The authors suggested that the diffuse staining might have been due to diffusion and absorption of enzyme from the circulating blood, since considerable 5′-nucleotidase activity could be demonstrated in the blood serum.

In the liver of rats treated with the carcinogen ethionine, the bile canaliculi stained very distinctly for 5′nucleotidase (Wachstein and Meisel, 1959). This pronounced staining, which was often greater than in normal liver, was observed at an early stage of ethionine treatment. In the cirrhotic phase the enzyme activity was evident in bile canaliculi, in most regenerating nodules. The staining of the sinusoids was irregular, whereas there was marked staining of sinusoids in the areas of the remaining liver cells, which were
mingled with oval cells. In carcinomatous areas there was no activity in the bile canaliculi, but a weak reaction in plasma membranes could be demonstrated. Much of the 5'-nucleotidase activity of hepatomas, studied cytochemically, appears to reside in connective tissue (Wachstein and Meisel, 1959; El-Aaser, 1965).

5'-Nucleotidase, with 5'-AMP as substrate, was demonstrated in Morris 5123, Dunning and Novikoff hepatomas [Novikoff (1960)]. Sinusoidal staining was observed in all three tumours. In the Morris hepatoma 5123 the bile canaliculi displayed remarkable activity. Some activity was possibly present in the Dunning hepatoma plasma membrane, but in the other tumours there was no activity in the plasma membranes.

In the early stage of carcinogenesis, induced in rat liver by 3'-methyl-4-dimethylaminoazobenzene and N-2-fluorenylacetamide, Chang (1960) observed an increase in the histochemical activity of 5'-nucleotidase and alkaline phosphate (with sodium glycerophosphate as substrate for the latter) and a decrease in the activities of adenosine triphosphatase and glucose-6-phosphatase.

In a biochemical study of a plasma membrane fraction from rat liver and hepatoma, Emmelot et al. (1964a) found some enzymic differences between the liver and hepatoma preparations. Using β-glycerophosphate as substrate, they found that the most notable difference was the presence of a rather strong alkaline phosphatase activity in the hepatoma membranes, which was absent in the liver preparations. With cytochemical techniques at the electron microscope level, Emmelot et al. (1964a) were unable to detect alkaline phosphatase activity in plasma membranes of liver in situ, but
found it to be abundantly present in those of hepatomas, this being in contrast with the observations of Novikoff (1960, 1962). Enzyme activity was also demonstrated in the microvilli of the plasma membrane and in the wall of some intracytoplasmic vacuoles.

A decrease in glucose-6-phosphatase activity assayed histochemically and biochemically, was noted in large hyperplastic nodules induced in rat liver with 2-fluorenylacacetamide or ethionine (Epstein, Ito, Merkow and Farber, 1967). The same authors presented evidence which implicated the induction of the hyperplastic nodules as a step prior to the appearance of malignancy. In accordance with earlier observations (e.g., those of Bernhard, 1958), an electron microscopic examination of the nodules revealed a decrease in parallel-arrayed rough endoplasmic reticulum and pronounced alterations of the smooth endoplasmic reticulum associated with a decrease in glucose-6-phosphatase activity, assayed biochemically (Merkow, Epstein, Caito and BaPtus, 1967).

8. Observations relating to membrane function

The functional aspect of membrane-bound enzymes is obviously important. Although electron microscopic studies often reveal mitochondria surrounded by cytomembranes, which may indicate some kind of co-operation in energy metabolism (Reid, 1967), there is no reason to believe that cytomembranes per se contribute largely to the energy supply of the cell. The supply of glucose to the bloodstream is controlled by glucose-6-phosphatase, an enzyme which is located in cytomembranes and whose location conceivably enables glucose to be channelled out of the cell (Reid, 1967).
As Quastel (1964) pointed out, there is substantial evidence that plasma membranes contain certain substances that play specific roles in conveying organic molecules from the cell exterior to the cell interior or from one compartment in the cell to another.

The participation of an 'ATPase' in the active transport of Na⁺ and K⁺ ions and its importance in nerve function is discussed by Conway (1960). In parenchymal cells of liver, ATPase activity is high in the microvilli of the bile canaliculi; the sinusoids also show some activity, in disagreement with Essner, Novikoff and Masek (1958). Moore and Ruska (1957) remark that the enzyme can safely be reckoned to play a significant role in 'active transport' across the plasma membrane and in cell movements such as pinocytosis and cytopempsis.

Ku and Wang (1963) observed that the concentration of DOC that activates 5'-nucleotidase is of the same order as the concentration in rat bile. Since 5'-nucleotidase activity is high in bile canaliculi, its activity in vivo may be under biliary control. De Verdier and Potter (see Novikoff, 1960) suggested that particularly in tumours the enzyme serves to furnish nucleic-acid precursors, in the form of nucleosides, to neighbouring cells, the plasma membrane being impermeable to nucleotides.

Ernster and Jones (1962) postulated that "The nucleoside diphosphatase may serve a function in promoting reactions involving the corresponding triphosphates as reactants by shifting the equilibrium toward the formation of the diphosphate ".

There has been much controversy as to whether RNA resides in the membranes isolated from the microsomal fraction or solely in ribosomes.
Campbell et al. (1964) considered that the RNA found in a membrane fraction isolated by Hawtrey and Schirren (1962) may have been due to contamination with ribosomes. Reid (1961a) and Petrovic, Becarevic and Petrovic (1965) found that when fractions which were rich in RNA supposedly derived from membranes were prepared from fasting rats given $^{14}$C-orotic acid or $^{32}$P-orthophosphate, the RNA labelling differed from that found for ribosome-rich fractions. The labelling tended to be relatively high soon after orotate administration, as if the RNA were of 'messenger' type. It is of interest that the properties of RNA in cytomembranes seem to resemble those of membrane-bound mitochondrial RNA (Roodyn, 1967).

When this work was initiated there was little evidence on the possibility that the plasma membrane may contain RNA, apart from the negative results obtained in autoradiographic experiments.
The aims of this study

El-Aaser et al. (1966b) partially separated 5'-nucleotidase-containing elements from those containing glucose-6-phosphatase by centrifuging a post lysosomal fraction in a 'B-IV' zonal rotor. The work reported here is an attempt to improve the separation of plasma membrane fragments (5'-nucleotidase as 'marker') from cytomembrane fragments (glucose-6-phosphatase as 'marker') by:

(1) Varying the time of zonal centrifugation, in the hope of achieving a good separation as the different membranes approached their isopycnic positions.

(2) Changing the shape of the gradient by using sigmoid gradients plateauing at density 1.17, as opposed to linear gradients. The use of sigmoid gradients should lessen the diffusion and overlapping of the different membrane types which occurs with linear gradients.

(3) Altering ionic conditions. Dallner (1963) separated smooth-surfaced cytomembranes from the rough-surfaced type in a rat liver microsomal fraction, by employing Cs⁺ and Mg⁺⁺ ions. The addition of these ions, to both the homogenization medium and the gradient, was exploited in the hope of attaining improved membrane separation.

(4) Varying the homogenization conditions. The separation of microsomal material, prepared from liver which was homogenized in hypotonic and isotonic sucrose and in saline, was investigated. The effects of deoxycholate on the separation of microsomal membranes, and also the effects of homogenization with a 'nitrogen' pressure homogenizer were studied.
In order to identify and characterize the various membrane fragments in a microsomal fraction, separated by zonal centrifugation, an electron microscopical (cytological) and cytochemical examination of the zonal fractions was carried out. A biochemical and morphological comparison was made between the membranous elements isolated from a liver microsomal fraction and those isolated from an ethionine-induced transplanted hepatoma microsomal fraction. It was hoped that such a comparison would yield information on the processes involved in carcinogenesis.

It is only in recent years that tissue fractions have been used in enzyme cytochemical studies, at least at the electron microscope level. Presumably this is because of the difficulty in handling such fractions. A method for preparing thin sections by layering liver homogenates on glass slides is reported in this thesis.

Fixation of tissue and incubation in media which often contain enzyme-inhibiting reagents are amongst the requirements of enzyme cytochemical techniques. A study has been made of the degree of enzyme inhibition due to fixation in glutaraldehyde - both commercial and purified - and to incubation in media containing varying concentrations of Pb²⁺ ions (used as 'capture' agents). One possible artefact in cytochemical work is an increase with time, in the number of membranous elements exhibiting cytochemical activity for 5'-nucleotidase. To check this, thin sections of microsomal material were incubated for varying periods of time. To check further possible artefacts due to non-specific deposition of lead phosphate, control assays in which the substrates were replaced with water were carried out in parallel with all cytochemical assays.

Plasma membrane fragments, isolated by zonal centrifugation of a crude nuclear fraction and a microsomal fraction, and also a crude microsomal fraction were cytochemically examined for 5'-nucleotidase
and glucose-6-phosphatase in an attempt to identify these plasma membrane fragments and to distinguish them from cytosome membrane fragments.

To aid in the characterization of a hepatoma induced by ethionine in this laboratory, an ultrastructural and cytochemical examination of the hepatoma was carried out. A crude nuclear fraction and a microsomal fraction from this hepatoma were also cytochemically examined in order to compare hepatoma enzyme localizations with those of normal liver.
CHAPTER XI

General Experimental Methods

1. Materials

'Analar' grade chemical reagents were used in all experiments. Enzyme incubation media, and media for tissue subcellular fractionation studies were prepared with glass distilled water. Values for pH refer to measurements at 18°.

Tris buffer tris(hydroxymethyl)amino methane, disodium salt of Adenosine-5'-monophosphate, Uridine-5'-monophosphate, Adenosine-5'-diphosphate, Uridine-5'-diphosphate, Adenosine-5'-triphosphate, Uridine-5'-triphosphate, and glucose-6-phosphate, barium salt of glucose-6-phosphate and Norit NX charcoal were purchased from Sigma Chemical Co. Ltd. Sodium β-glycerophosphate, sodium cacodylate and osmic acid were purchased from British Drug Houses Ltd. Sodium deoxycholate was obtained from Hopkin & Williams Ltd. Glutaraldehyde (25% in water) and β, β-dimethyl-glutaric acid were purchased from Koch-Light Ltd. Kodak Bromide papers were used in the printing of all the electron micrographs.
Animals

Hooded rats, weighing about 250 g., of a Chester Beatty Research Institute strain were used throughout the study. Rats from this strain were delivered by Caesarian section and maintained for several generations in the Specific Pathogen Free Unit at Allington Farm, Ministry of Defence, Porton Down, Salisbury. From these a breeding nucleus was obtained for the University of Surrey, where the rats have been kept under conventional conditions. As far as can be elucidated they have maintained their Specific Pathogen Free status after three years of random breeding.

The hepatomas studied were transplants of a primary hepatoma (denoted 'U'; with sublines 'UA' or 'UB') which had been induced by ethionine. Rats were fed ethionine over a period of 7 months, there being a break after the first 5 weeks of feeding and 3 other breaks amounting to a total of 5 weeks. The original hepatoma arose 8 months after ethionine feeding had ceased and was maintained by a subcutaneous transplantation - usually every 2-3 1/2 weeks. With progressive transplantation of the hepatoma, which had a fast growth rate, the level of glucose-6-phosphatase activity decreased to one-fourth of that found in normal liver. For most experiments, rats fasted for a period of 17 hours were used.
3. **Preparative methods for tissue fractions**

The fractionation procedures employed were based on those of de Duve, Pressman, Gianetto, Wattiaux and Appelmans (1955). Livers from male rats, which were about 12 weeks old, were used in most experiments. Unless otherwise stated the homogenization and suspension medium was 0.25 M sucrose.

(a) **Tissue fractions for zonal centrifugation**
Weighed liver from rats, killed by cervical dislocation, was chopped into small pieces in ice-cold 0.25 M sucrose. The tissue was homogenized in nine volumes of the same medium with 10 up and down strokes of a Potter-Elvehjem homogenizer (A. Thomas Inc.), using a pestle rotating at 2,000 r.p.m. All preparative work was carried out at 4°C. The homogenate was centrifuged at 12,000 r.p.m. (15,000 g max) for 20 minutes, in a 'High Speed 18' M.S.E. centrifuge using an 8 x 50 ml. angle rotor to remove large cell organelles. The supernatant, after a transportation delay, was loaded into the 'B IV' rotor of a zonal centrifuge, [Unlike work in this laboratory on the separation of nuclear fraction components on an 'A' rotor centrifuge, the delay in loading seemed to have no effect when applied to the separation of microsomal material on the 'B IV' rotor.]

(b) **Operational techniques of zonal centrifugation**
The 'B IV' rotor was filled with gradient during rotation at a low speed (c 4,000 r.p.m.). The light end of the sucrose gradient, formed on a Beckman gradient maker, was pumped to the rotor wall, followed by denser sucrose which displaced the lighter end of the gradient towards the rotor core. When the gradient was in
the rotor, an 'underlay' of dense sucrose was pumped in until the light end of the gradient began to flow out of the central line. At this point the microsomal sample was introduced through the centre line, reversing the direction of fluid flow through the rotor and causing part of the underlay to flow back out through the edge line. To push the sample layer further into the rotor chamber clear of the core, an 'overlay' of low density sucrose was pumped in. The connection to the rotor edge was then closed and the centre line attached to a reservoir of water to allow a small volume of fluid to flow into the rotor during acceleration to compensate for rotor expansion. The rotor was then accelerated to a speed of 40,000 r.p.m. and maintained at this speed for times varying from two to nine hours.

After deceleration to low speed, the gradient was displaced by a solution of dense sucrose towards the core and out through the centre line. The gradient flowed through an ultraviolet absorbance monitor and 40 ml. samples were collected in cooled tubes. The sucrose density was estimated from the refractive index of each sample.

In most cases the sucrose gradient was buffered with 0.005 M Tris pH 7.4, to reduce the risk of artefacts due to aggregation of particles, in the presence or absence of magnesium or erbium ions. In run 25 the gradient was adjusted to pH 7.4 with sodium bicarbonate.

(c) **Tissue fractions for enzyme inhibition studies and cytochemical studies**

Liver from male rats was homogenized in 0.25 M sucrose [Chapter II (a)] and filtered through a nylon sieve of coarse mesh to remove connective tissue. A portion of the homogenate (Horn.) was left aside for enzyme studies. The remainder was centrifuged
at 15,000 g for 20 minutes in a 'High Speed 18' M.S.E. centrifuge using an 8 x 50 ml. angle rotor. The resulting pellet (termed 15,000 g pellet), which contains nuclei, mitochondria and lysosomes, was stored at 4°. The supernatant was diluted with an equal volume of 0.25 M sucrose and centrifuged at 55,000 g for 90 minutes in a 'Superspeed 50' M.S.E. centrifuge using a 10 x 100 ml. angle rotor. The pelleted material was homogenized with a Potter homogenizer in the same medium, yielding a microsomal fraction (Mic.), 10 ml. of which was equivalent to 1 g. of original liver.

For lead inhibition studies small aliquots (0.1 ml.) of the whole homogenate (Hom.) and the microsomal fraction (Mic.) were taken and incubated in the appropriate enzyme medium.

For glutaraldehyde inhibition studies the 15,000 g pellets and the microsomal pellets were fixed in varying concentrations of the aldehyde for 2 hours and washed in 0.25 M sucrose buffered at pH 7.4 with 0.2 M sodium cacodylate. The fixed pellets were homogenized in unbuffered 0.25 M sucrose and aliquots biochemically assayed for 5'-nucleotidase and glucose-6-phosphatase.
4. Chemical estimations

(a) Protein

Protein was estimated according to the method of Lowry, Rosenbough, Farr and Randall (1951). Reagent C, which consists of 50 parts of a solution of 2% Na₂CO₃ and 1 part of 0.5% CuSO₄·5H₂O in 10% Na or K tartarate, was prepared and mixed on the day of use. To 0.5 ml. zonal fractions, 0.5 ml. of 1 N NaOH was added and the mixture incubated at 37° to facilitate solubilization. The solution was cooled to room temperature, 5 ml. of reagent C was added, and the mixture was shaken. After 15 minutes, 0.5 ml. of Folin-Ciocalteu phenol reagent was added and the tubes were immediately shaken. The intensity of the blue colour, which was allowed to develop for 30 minutes, was read at 750 μm on a Unicam spectrophotometer. Solutions containing 100 μg. and 200 μg. of serum albumin per ml. were used as standards. The results are expressed as mg. protein per tube.

(b) RNA

Zonal samples were precipitated, using 2.5 mg. of casein as 'carrier', with an equal volume of cold 10% PCA, and centrifuged. The pellets were washed several times with 5% PCA and defatted twice at room temperature with a mixture of alcohol, ether and chloroform (2:2:1). The dried pellets were extracted, twice for 15 minutes, with 5% PCA at 80°. A solution of 0.5% orcinol in conc. HCl plus CuCl₂ 0.007% was added to the extract. After heating at 95° for 30 minutes, the intensity of the green colour was measured at 665 μm on a spectrophotometer.
5. **Enzyme assays**

In most cases enzymes were assayed by methods based on those of de Duve *et al.* (1955) with slight modifications [El-Aaser, 1965].

(a) **5'-Nucleotidase**

0.35 ml. 0.25 M Tris, pH 8.0
0.05 ml. 0.05 M MgCl₂
0.1 ml. 0.05 M 5'-AMP or 5'-UMP
0.5 ml. zonal fraction

(b) **Glucose-6-phosphatase (G-6-Pase)**

0.3 ml. 0.2 M dimethylglutarate, pH 6.5
0.1 ml. 0.1 M EDTA
0.1 ml. 0.05 M G-6-P
0.5 ml. zonal fraction

(c) **Nucleoside diphosphatase**

0.35 ml. 0.25 M Tris, pH 8.0
0.05 ml. 0.05 M MgCl₂
0.1 ml. 0.025 M UDP or ADP or IDP
0.5 ml. zonal fraction

(d) **Nucleoside triphosphatase**

0.35 ml. 0.25 M Tris, pH 8.0
0.05 ml. 0.05 M MgCl₂
0.1 ml. 0.025 M ATP or UTP
0.5 ml. zonal fraction

(e) **Inorganic pyrophosphatase (PPase)**

0.3 ml. 0.25 M Tris, pH 8.0
0.1 ml. 0.05 M MgCl₂
0.1 ml. 0.05 M Na₃pyrophosphate
0.5 ml. zonal fraction
(f) **Alkaline phosphatase**

0.4 ml. 0.2 M glycine, pH 9.6

0.1 ml. 1 M β-glycerophosphate (diNa salt)

0.5 ml. zonal fraction

The phosphate was estimated by the method of Berenblum and Chain (1938).

(g) **Acid phosphatase**

0.4 ml. 0.3 M dimethylglutarate, pH 4.6

0.1 ml. 0.1 M p-nitrophenylphosphate

0.5 ml. zonal tissue [freeze-thawed about 5 times to release bound enzyme activity (de Duve et al., 1955)].

Between 1 - 2 mg. was used in the case of tissue from the whole homogenate (Hom.) and the microsomal fraction (Mic.).

All incubations were carried out at 37° for times varying from 15 minutes to 2 hours. The reaction was stopped by adding 1.5 ml. 6% TCA. The mixture was centrifuged and the supernatant transferred to clean tubes. The inorganic phosphorous was determined according to Lowry and Lopez (1946) - 1.5 ml. 0.6% Ammonium molybdate in 5% PCA was added to the supernatant and the phospho-molybdate complex reduced with 0.5 ml. (1 mg.) ascorbic acid. The intensity of the blue colour was read either at 720 mμ or at 750 mμ on a Unicam spectrophotometer, or at 700 mμ on an 'EEL' colorimeter, after 30 minutes' developing time. The enzyme activities are expressed as μ moles phosphorous released/minute/tube, after tissue and substrate blanks had been subtracted from the experimental readings. The release of phosphate from 5'-AMP and from G-6-P was linear for at least 1 hour's incubation.
Later in the study a Technicon Autoanalyser was used for the determination of inorganic phosphate (M.T. Marzban, unpublished methods) and of protein by an adaptation of a Lowry technique (Schuel and Schuel, 1967). The method used for the estimation of inorganic phosphate was adapted from the manual procedure of Lowry and Lopez (1946).

6. Processing of results

Sucrose is known to interfere with the assays of protein by the Lowry method (Schuel and Schuel, 1967) and the assays of many enzymes (de Duve et al., 1955; El-Anser, 1965). Therefore the values for protein and for enzyme activities were corrected for inhibition by sucrose following the procedure of Hinton, Burge and Hartman (1969). The total enzyme activity in each zonal fraction was calculated and ratios were produced using the program of Hinton (unpublished methods). In the same program the enzyme activities were recalculated to be linear with the density of the gradient. The method employed was simply to divide the values for the protein and enzyme activities in each fraction by the difference in the density of the medium between the 'beginning' and 'end' of that fraction.

7. Purification and estimation of glutaraldehyde

Following the method of Anderson (1967), about 200 ml. of 25% aqueous commercial glutaraldehyde was added to 30–40 g. of Norit NK charcoal in a glass beaker. After thorough mixing the slurry was vacuum filtered through Whatman No. 44 filter paper, mounted in a Buchner funnel. Remixed with charcoal and filtering was repeated 4 times and the purified glutaraldehyde stored in a dark bottle at 4°C.
The percentages of glutaraldehyde in the commercial and purified solutions were calculated when the glutaraldehyde solutions were estimated by a chemical method (Anderson, 1967).

8. **Cytochemical and electron microscopical techniques**

(a) General techniques

In most cases the technique of Holt and Hicks (1961), with slight modifications (El-Aaser, 1965), was used because the literature survey outlined in the Introduction showed it to be a good technique.

In some cytochemical studies, liver and hepatoma sections were fixed in glutaraldehyde. Liver or hepatoma was removed from a rat and immersed in 4% glutaraldehyde, at 4°C, buffered with 0.1 M sodium cacodylate pH 7.4. The tissue was chopped with a scalpel blade into small cubes of about 1 mm³, and fixed for 3 hours. It was then washed in 0.25 M sucrose buffered at pH 7.4 with 0.1 M sodium cacodylate and stored in ice-cold gum sucrose (0.88 M sucrose containing 1% gum acacia). Some fixed tissue was transferred, by means of a dropper, to the platform of an M.S.E. freezing microtome and sections, 30μ thick, were cut. Sections were then incubated in the appropriate enzyme medium and shaken at intervals. After sufficient incubation, judged by the formation of a black precipitate of lead sulphide upon exposure of the sections to Na₂S, the medium was decanted and the sections washed several times with 0.25 M sucrose and finally with distilled water. The tissue was the post-fixed in 1% osmium tetroxide for 1 hour and dehydrated by 10-minute periods of exposure in 30%, 50%, 70% and (twice) 100% ethyl alcohol. It was then immersed in propylene oxide for 10 minutes and embedded, usually the same day, in araldite for 48 hours at 60°C. Ultrathin sections, with silver or gold interference colours were cut on an LKB ultratome,
using a glass knife, and examined in an A.E.I. 6G electron microscope. In some cases sections were counter-stained with alkaline lead hydroxide (Karnovsky, 1961) before electron microscopic examination.

In morphological studies fixation in glutaraldehyde was omitted and liver blocks (1 mm$^3$) or pellets, which were very small in size, were fixed in 1% osmium tetroxide and processed for electron microscopy (see above).

(b) Cytochemistry on fractions

In the cytochemical investigation of tissue fractions, 3 methods for preparing thin sections were employed. The merits of such methods are discussed later in this thesis. In the first (Method 'A'), a viscous suspension of microsomal material was obtained by homogenizing a crude microsomal (15,000 g) pellet in concentrated sucrose solutions - up to 1 M. The suspension, which in some instances was fixed in glutaraldehyde, was layered onto glass microscope slides which were tilted to allow the formation of a thin film. The tissue was then dried under a stream of air at 4° for between 4 and 12 hours. Using a scalpel blade thin sections were peeled off the slides, incubated in the enzyme media and processed for electron microscopy.

In the second method (Method 'B') a crude microsomal pellet was frozen and 30μ sections, which were cut on a freezing microtome, were incubated to demonstrate cytochemical enzyme activity. In some instances the pellet was pre-fixed in varying concentrations of glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.4.
Purified membrane fractions, obtained by zonal centrifugation of hepatic nuclear and microsomal material, were employed in some cytochemical studies. The membranous material was centrifuged and thin sections were obtained without cutting the pellet. In this third method (Method C!) the extremely small pellet was flattened against the side of the centrifuge tube with a glass rod to give an ultrathin preparation which was then dislodged. It was incubated in situ with appropriate enzyme media and finally tipped into a glass test tube and processed for electron microscopy.

(c) The media for cytochemical assays

The incubation media for 5'-nucleotidase and for adenosine triphosphatase were based on those of Wachstein and Meisel (1957) and the medium for glucose-6-phosphatase on that of Tice and Barrnett (1962), with some modifications by El-Aaser (1965). Dimethylglutarate buffer, whose pH was adjusted with NaOH was used. The use of this buffer was found to prevent the spontaneous precipitation of lead from the incubation media (El-Aaser and Birbeck, unpublished experiments). The possibility that Na⁺-K⁺-Mg⁺⁺-activated adenosine triphosphatase is being examined cannot be ruled out because of the presence of Na⁺ ions in the incubation medium.

5'-Nucleotidase

10 ml. 0.2 M dimethylglutarate, pH 7.2
8.5 ml. distilled water
2.5 ml. 0.1 M magnesium nitrate
2.5 ml. 0.05 M 5'-UMP (dINa salt)
1.5 ml. 2% lead nitrate
Glucose-6-phosphatase

11.0 ml. distilled water
4.0 ml. 0.02 M G-6-P • (diNa salt)
4.0 ml. 0.03 M dimethylglutarate, pH 6.5
1.5 ml. 2% lead nitrate

Adenosine triphosphatase

11.0 ml. 0.2 M dimethylglutarate, pH 7.2
5.0 ml. distilled water
2.5 ml. 0.1 M magnesium nitrate
5.0 ml. 0.005 M ATP (13 mg. of ATP, diNa salt)
1.5 ml. 2% lead nitrate

Media in which the substrates were replaced by water were used as controls.
A. Zonal centrifugation of hepatic microsomal material in a 'B IV' rotor.

1. Marker enzymes

Liver sections were cytochemically examined, at the electron microscope level, in order to furnish information on the intracellular localization of certain phosphatase enzymes. 5'-Nucleotidase activity, as judged by lead phosphate deposition (with UMP as substrate), was demonstrated in the plasma membrane especially where it folds to form bile canaliculi (Fig. 2). Enzyme activity, although not as pronounced as that demonstrated in bile canaliculi, was also demonstrated in the microvilli that form the blood sinusoidal wall but no activity was observed in mitochondria, the nucleus or nuclear envelope or in the membranes of the endoplasmic reticulum (ER). Activity was demonstrated in lysosomes, this being due to the presence in lysosomes of a non-specific acid phosphatase. These observations are in agreement with those of Essner et al. (1958), Sabatini et al. (1963) and El-Aaser (1965).

Glucose-6-phosphatase (G-6-Pase) activity was demonstrated in the ER membranes, both rough-surfaced and smooth-surfaced, and in the nuclear envelope [Fig. 3 (a) and (b)] but not in mitochondria or in the plasma membrane [see Fig. 3 (b)].

When a liver section was incubated for adenosine triphosphatase (ATPase) a heavy deposit of lead phosphate was observed in the plasma membrane and in the microvilli of the sinusoidal wall [Fig. 4 (a) and (b)] in agreement with the observations of Essner et al. (1958), Novikoff et al. (1958), Ashworth et al. (1963), Sabatini et al. (1963) and El-Aaser (1965). No enzyme activity was demonstrated within or on the surface of mitochondria, in the ER membranes or in the
nucleus or nuclear envelope. Deposition of lead phosphate on a red blood cell membrane is shown in Fig. 4 (a).

Following these cytochemical observations, 5'-nucleotidase was employed as an enzyme marker for membrane fragments derived from the plasma membrane and G-6-Pase as a marker for ER membranes in work on zonal centrifugation of hepatic microsomal material.

Legends to Figs. 1-4

Fig. 1  Electron micrograph of liver section showing nucleus (N), nuclear envelope (NE), endoplasmic reticulum (ER), mitochondria (M), bile canaliculi (BC), lysosome (L), sinusoid (S), microbody (m) and lipid (l).

Lead stained.
Magnification x 7,500

Fig. 2  5'-Nucleotidase, 5'-UMP as substrate. Activity is seen at BC and L but not at N, NE, M or ER. Incubation 20 minutes at 37°.
Electron micrograph x 18,000

Fig. 3  G-6-Pase. Deposits seen at ER and NE, no activity in N, M or PM. Incubation 20 minutes at 37°.
Electron micrograph x 30,000

Fig. 4  ATPase. Heavy staining in PM and at S, no staining in ER, N, NE or M. Incubation 20 minutes at 37°.
Electron micrograph x 30,000
Fig. 1
Fig. 2
2. **Effects of varying homogenization medium or time of zonal centrifugation**

For the purpose of studying the distribution of enzyme activities the gradient was divided into three regions. The first, that area of the gradient covering approximately the first ten fractions, corresponds to the 'soluble' fraction of a liver homogenate. It will contain both small ions and large molecules having a sedimentation coefficient of less than 20. Normally this region corresponded to the region of the gradient having a density of less than 1.10. In the second or 'central' region the density of the gradient ranges from 1.10 to 1.17. An electron microscopic examination of this region, to be presented later, revealed membrane fragments of a smooth-surfaced type - both plasma membranes and cytomembranes. The third region, where the density of the gradient is greater than 1.17, is rich in RNA and on electron microscopic examination consists mainly of rough-surfaced cytomembranes.

The starting material in the present work was the 'post-lysosomal supernatant' [Chapter II, 3 (a)], henceforth termed 'microsomal material'. For the conditions employed in zonal runs see Table 1.

(a) **Homogenization in 0.12 M sucrose**

In this laboratory success was achieved in the isolation of plasma membrane fragments from a crude nuclear fraction prepared by initial homogenization of liver in hypotonic sucrose. Good results were achieved with 0.12 M sucrose which seemed to cause less adhesion of plasma membrane fragments to nuclei than either isotonic or hypertonic sucrose.
When 'microsomal material' was prepared by homogenization in 0.12 M sucrose, varying the time of zonal centrifugation from 3 hours to 9 hours had no noticeable effect on the amount of 5'-nucleotidase (AMPase) recovered in the region of density ranging from 1.10 to 1.17. [Table 1, Runs 33, 30 and 29 (in run 34, there were variables besides time, as will be considered later)]. This observation established that isopycnic conditions were attained even within 3 hours. Fig 5 (runs 33 and 34) shows the distribution pattern of AMPase and G-6-Pase in a microsomal preparation (the original homogenate having been made in 0.12 M sucrose) centrifuged for 3 hours in a 'B IV' zonal rotor. The shape chosen for the gradient was similar in both runs, being sigmoid and plateauing at a density of 1.17. As can be seen from Table 1 the bulk of the AMPase activity in run 33 was recovered in the density region 1.10 < D_{20} < 1.17 and that of G-6-Pase in the region 1.18 < D_{20} < 1.23. If the concentration of Mg++ was increased from 0.005 M (run 33) to 0.1 M (run 34) the density of the plasma membrane fragments increased and AMPase was almost evenly distributed between the two density regions while G-6-Pase was mainly in the region of higher density (D_{20} > 1.17).

The distribution pattern for RNA paralleled closely that for G-6-Pase. The levels of enzyme activities in the 'soluble' region (usually the first ten fractions) were generally quite variable. El-Aaser (1965) centrifuged material isolated from the 'soluble' region in a similar type zonal run and obtained a small sediment containing the bulk of the AMPase activity but lacking membranous structures when examined under the electron microscope.
### Table 1

Distribution of phosphatase enzymes amongst zonal fractions run in a 'B-IV' rotor

<table>
<thead>
<tr>
<th>Homogenization Medium</th>
<th>0.12M Sucrose</th>
<th></th>
<th></th>
<th>0.25M Sucrose</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of Centrifugation</td>
<td>3 hr. 8 hr. 9 hr. 3 hr. 5½ hr. 6 hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run No.</td>
<td>33 34 30 29</td>
<td>38 41 35 19 18 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg²⁺ concentration</td>
<td>0.005M 0.01M 0.005M 0.005M 0.005M</td>
<td>.005</td>
<td>.004M 0.005M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cs⁺ concentration</td>
<td>- - - - - 0.01M</td>
<td>- - 0.005M</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deoxycholate (DOC)</td>
<td>- - - - - -</td>
<td>- - - - - 0.5% 0.25%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>- - - - - -</td>
<td>- - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris 0.005M</td>
<td>+ + + + + +</td>
<td>+ + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.5 8.0 7.4 7.4</td>
<td>7.4 7.4 7.4 7.4 7.4</td>
<td>7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPase % zonal activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D₂₀ &lt; 1.10</td>
<td>23% 17% 14.5% 12.5%</td>
<td>22% 19% 15% 22% 59% 38%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.10 &lt; D₂₀ &lt; 1.17</td>
<td>60% 40% 58% 55%</td>
<td>55% 73% 48% 57% 24.5% 52.5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D₂₀ &gt; 1.17</td>
<td>17% 43% 27.5% 32.5%</td>
<td>23% 8% 37% 21% 16.5% 9.5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-6-Pase % zonal activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D₂₀ &lt; 1.10</td>
<td>3% 11% 9%</td>
<td>7.5% 8.5% 3.5% 13% 3% 5.5% 18.5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.10 &lt; D₂₀ &lt; 1.17</td>
<td>23% 15% 22% 24.5% 33% 69% 33% 58% 37.5% 58.5%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D₂₀ &gt; 1.17</td>
<td>75% 74% 69% 68%</td>
<td>58.5% 27.5% 54% 39% 57% 23%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Total activity of fractions recovered from the zonal rotor taken as % recoveries typically found for enzyme activities loaded into rotor:

- 5' Nucleotidase = 73%
- G-6-Pase = 68%
Microsomal material from 2.7 g liver was loaded.

Microsomal material from 5 g liver was loaded.

Run No. 34

Fig. 5
In run 29 where the gradient was of a linear type, the enzyme activities are spread over a larger number of fractions than in run 30 where, with a sigmoid gradient, better separation of AMPase from G-6-Pase is achieved. In run 30, which is somewhat similar to that of run 29, the distribution of Mg\(^{++}\)-activated ATPase follows quite closely that of AMPase and the distribution of RNA parallels that of G-6-Pase (Fig. 6).

(b) **Homogenization in 0.25 M sucrose**

Since Hogeboom, Schneider and Palade (1948) introduced sucrose as a medium in tissue fractionation procedures and since high concentrations of sucrose inhibited certain metabolic processes, 0.25 M (isotonic) sucrose became the medium most commonly used in such fractionation procedures (Schneider and Hogeboom, 1951; Hogeboom, Kuff and Schneider, 1957).

In comparing runs 30 and 29, in which the initial homogenization was in 0.12 M sucrose, with runs 26 and 27, where the homogenization medium was 0.25 M sucrose, little difference in the distribution of phosphatase enzymes amongst the different density regions was encountered (Table 1).

Fig. 7 shows the enzyme distribution pattern in 'microsomal material', prepared by initial homogenization in 0.25 M sucrose, and centrifuged for 6 hours. Besides a peak of enzyme activities in the 'soluble' region two main peaks were again observed, 5'-Nucleotidase (the substrate being UMP in this run) was located in a region containing smooth membranes (see below) ranging in density from 1.10 to 1.17, relatively free from G-6-Pase (Table 1) which was located at a higher
Microsomal material from 5 g. liver was loaded.

FIG. 6

Run No. 30
Microsomal material from 4.7 g. liver was loaded.
density ($c \rho = 1.2$). The distribution pattern of the diphosphatase enzymes is considered later. As with other runs the distribution of RNA parallels that of G-6-Pase and the distribution of Mg$^{++}$-activated ATPase parallels that of 5'-nucleotidase.

To obtain information on the morphology of the zonal fractions, material from some of these fractions was centrifuged and the pellets processed for electron microscopy. Fig. 8 (a) is an electron micrograph of material from fractions 20 and 21 in run 32 (Fig. 7). It shows vesicles (mainly smooth-surfaced but with some rough-surfaced also present) and sheets of membrane. Many of the sheets exhibit paired membrane features. Fig. 8 (b), an electron micrograph of material from fractions 24 to 26 in the same run, shows a rather large sheet of membrane (bottom left) along with many smaller sheets and some vesicles. Fig. 8 (c) shows material from fractions 34 and 35 (also run 32) consisting of smooth-surfaced and rough-surfaced vesicles and sheets of membrane. Membrane fragments consisting mainly of a rough-surfaced type, both vesicular and tubular were observed in an electron micrograph of material from fractions 38 and 39 [Fig. 8 (d)].
Legends to Figs. 8 and 9

Fig. 8  Electron micrographs of pellets from zonal centrifuge fractions in run 32 (see Fig. 7)

(a) Fractions 20-21 showing some sheets of membrane and vesicles, mainly smooth-surfaced but with some rough-surfaced also present. Lead stained.
Magnification x 30,000

(b) Fractions 24-26 shows a large sheet of membrane (lower left) and many smaller sheets. Lead stained.
Magnification x 30,000

(c) Fractions 34-35 showing some sheets and smooth- and rough-surfaced vesicles. Lead stained.
Magnification x 30,000

(d) Fractions 38-39 showing mainly rough-surfaced membrane fragments. Lead stained.
Magnification x 30,000

Fig. 9  Electron micrographs of pellets from two bands obtained be recentrifugation to equilibrium of zonal fractions from the central region of run 35 (see Table 1).

(a) and (b) Different sections of a pellet from upper band, rich in enzyme activity and RNA, showing some sheets of membranes and vesicles. Lead stained.
Magnification x 30,000

(c) Section of pellet from lower band, lacking enzyme activity and RNA and containing vesicles. Lead stained.
Magnification x 30,000
From this ultrastructural examination of zonal fractions it can be seen that material from under the 5'-nucleotidase peak consists mainly of smooth-surfaced membranes and that rough-surfaced membranes are isolated in fractions having a high G-6-Pase activity and also containing most of the RNA of the microsomal material loaded into the zonal rotor. However a distinction cannot be made between plasma membrane fragments (containing 5'-nucleotidase) and smooth-surfaced cytomembranes (containing G-6-Pase) at least on a morphological basis. A cytochemical examination of zonal fractions is considered later in this thesis.

In runs 26 and 27 the time of zonal centrifugation was increased to 9 hours to ensure isopycnic conditions (see Fig. 10). There was a slight migration of G-6-Pase to a region of higher density but very little difference was observed in the location at different densities of 5'-nucleotidase when compared with 3 hour or 6 hour runs (Table 1). It thus seems likely that both enzymes have reached isopycnic positions after 3 hours of zonal centrifugation. In run 27, AMPase, although relatively free from G-6-Pase, is distributed over a wide number of fractions (Fig. 10). In spite of the steeper gradient in run 26 (rising to a density of 1.13 at fraction 9 as compared to fraction 14 in run 27) no improved separation of enzymes was achieved. In run 27 an electron microscopic examination (not illustrated) revealed smooth-surfaced membrane sheets and vesicles in fractions 12 to 18 and 24 to 29, whereas fractions 36 to 38 contained mainly rough-surfaced membranes and free ribosomes.
Microsomal material from 4 g. liver was loaded

Run No. 27

Run No. 26

'Microsomal material' from 4 g. liver was loaded
Zonal fractions were recentrifuged on sucrose gradients in an attempt to achieve further purification. When fractions taken from the different regions of the gradient in run 35 (see Table 1) were centrifuged for 13 hours in a swing-out rotor on a continuous sucrose gradient (ranging in molarity from 1 M to 2 M) most of the fractions gave a single band. However, material from the 'central' region \(1.10 < D_{20} < 1.17\) separated into two bands. The upper band \(D_{20} = 1.18\) contained all the enzyme activities - Mg-activated ATPase, AMPase and G-6-Pase - and all the RNA of the region. On electron microscopic examination this band contained many vesicles and some sheets of membrane [Fig. 9 (a) and (b)]. The lower band \(D_{20} = 1.29\) exhibited little or no enzyme activity and consisted mainly of vesicular material [Fig. 9 (c)] similar to that found in the upper band.

(d) Homogenization in 0.14 M NaCl

It was pointed out by de Duve (1967) that sucrose solutions used in homogenization media are not always superior to ionic media, as was originally believed (de Duve and Berthet, 1954). In run 31 (Fig. 11), where the homogenization medium was 0.14 M NaCl instead of sucrose, an improved separation of AMPase from G-6-Pase was achieved. The time of zonal centrifugation was 6 hours. As can be seen from Table 1 and Table 2a (see below) there was an increase in activity for 5'-nucleotidase and other phosphatase enzymes in the 'soluble' region. It seems that NaCl causes the release of phosphatase enzymes into the soluble region. A sharp peak for AMPase was observed at a density of 1.12, the level of G-6-Pase in this region being quite low (see Fig. 11). Sharp peaks for RNA in fractions 18 and 23 with
Microsomal material from 5 g. liver was loaded.

Fig. 11
Fig. 11 (CONTINUED)
corresponding low activities for G-6-Pase suggest the possibility that NaCl strips ribosomes (the main sources of RNA) from cytomembranes. Ribosomes would be sedimenting in this region under the conditions used.

An electron microscopic examination (not illustrated) of different fractions from run 31 (see Fig. 11) revealed the presence of small sheets of membranes in fraction 14. Fractions 17 and 18 contained small sheets of membrane and free ribosomes. Smooth-surfaced vesicles, sheets of membrane and free ribosomes were observed in fractions 22 to 24 while fractions 37 and 38 contained rough-surfaced vesicles.

3. Effects of cations

When Mg++ ions were omitted from the homogenization medium and from the gradient, most of the AMPase activity was recovered in association with G-6-Pase in the 'central' region (see Table 1). Fig. 12 illustrates the distribution patterns for enzymes and for RNA in 'microsomal material' prepared and centrifuged (3 hours) in the absence of Mg++ ions. As can be seen, separation of AMPase from G-6-Pase was not accomplished.

In run 25, Mg++ ions were omitted from the homogenization medium and from the gradient, which was adjusted to pH 7.5 with sodium bicarbonate, rather than with Tris buffer. The phosphatase enzymes examined were distributed over a large number of tubes and it was not possible to separate AMPase from G-6-Pase (Fig. 13). Peaks for RNA coincide closely with those for G-6-Pase and Mg++-activated ATPase activity parallels that of AMPase. Activities for inorganic pyrophosphatase (Mg++-activated) and acid p-nitrophenyl-phosphatase were almost entirely in the 'soluble' region.
Microsomal material from 3.8 g. liver was loaded
Microsomal material from 4 g. liver was loaded
Fig. 13 (continued)
Good separation of AMPase from G-6-Pase was achieved only in the presence of Mg\textsuperscript{++} ions (see Table 1). In most runs with Mg\textsuperscript{++} present, the concentration was 0.005 M. Increasing the concentration to 0.01 M caused no marked change in the positions of the enzyme peaks (see Fig. 5).

Run 19 was carried out in the presence of Cs\textsuperscript{+} (0.005 M) in place of Mg\textsuperscript{++}. No separation of AMPase from G-6-Pase was achieved (Fig. 14). As can be seen from Table 1, in a run with Cs\textsuperscript{+} ions present (run 19) equal amounts of AMPase and G-6-Pase were recovered in the 'central' region.

The enzyme distribution pattern obtained in the presence of Mg\textsuperscript{++} ions (0.005 M) together with Cs\textsuperscript{+} (0.01 M) is shown in run 38 (Fig. 14). In this run, the time of zonal centrifugation was 3 hours. Reasonable separation of AMPase from G-6-Pase was obtained but no marked improvement over that employing Mg\textsuperscript{++} alone was observed.

4. Treatment with deoxycholate (DOC)

When used at concentrations lower than those used to solubilize membranes, sodium deoxycholate is known to detach ribosomes from cytomembranes and has been used in attempts to separate smooth-surfaced elements from rough-surfaced elements (Ernster, Siekevitz and Palade, 1962). 5'-Nucleotidase can also be solubilized from smooth membrane fragments by means of DOC (Song and Bodansky, 1966 and 1967).

Fig. 15 shows the enzyme distribution pattern for 'microsomal materials'(prepared by initial homogenization in 0.25 M sucrose) treated with DOC to 0.5% (run 18) or to 0.25% (run 20) and centrifuged for 6 hours.
Microsomal material from 4 g. liver was loaded.

Microsomal material from 5 g. liver was loaded.

Run No. 19
Microsomal material from 5 g. liver was loaded.

Fig. 15
AMPase, which may be no longer associated with membranes, has shifted to a region of low density (soluble region). It seems as if the enzyme is loosely bound to the membrane and is easily detached by deoxycholate.

More than 50% of the G-6-Pase activity was recovered at a density greater than 1.17 (run 18). This indicates that the enzyme is more firmly bound to the membrane than is AMPase, which is in agreement with the work of El-Aaser (1965) who showed that with microsomal fractions, 5'-nucleotidase is more readily solubilized by DOC than is G-6-Pase.

As can be seen from run 18 (Fig. 15 and Tables 1 and 2) DOC at a final concentration of 0.5% causes the release of about 50% of the ATPase activity into the 'soluble' region. However, 40% of the ATPase activity was recovered in the 'central' region of the gradient, as against 24% for 5'-nucleotidase, indicating that ATPase is a more firmly membrane-bound enzyme than 5'-nucleotidase (Dallner, 1963).

5. Homogenization with a 'nitrogen pressure homogenizer'

Microsomal material, prepared from a parenchymal cell suspension which was homogenized in a 'nitrogen pressure homogenizer', was separated by centrifugation for 2 hours in a 'B IV' rotor. (The conclusions from this experiment should be interpreted with caution since the use of a cell suspension, as against whole liver, introduced a second variable.)

Fig. 16 shows the enzyme distribution pattern for such a zonal run (run 237). The peak representing AMPase activity appears to be quite sharp and a reasonably good separation from G-6-Pase was achieved.
Microsomal material from 5 g. liver was loaded
Such vigorous homogenization conditions might be expected to cause severe fragmentation of membranes with an increase in the recovery of such membranous elements in the microsomal fraction. Confirming this, Hinton, Klucis, El-Aaser, Fitzsimons, Alexander and Reid (1967) found that by using the 'nitrogen pressure homogenizer' there was a fall in the normally large amount of 5'-nucleotidase recovered in the crude nuclear fraction.

6(a) Distribution of nucleoside di- and triphosphatases amongst zonal fractions.

In a cytochemical examination of rat liver, carried out at the level of the electron microscope, El-Aaser (1965) noted UDPase activity in the plasma membrane and in the nuclear envelope and cytomembranes (ER). As can be seen from Table 2a, activity for UDPase is largely in the soluble region. A peak for UDPase activity was found at a density in the region of 1.15 to 1.17 (runs 19, 25, 31 and 32), this being the region containing the bulk of the 5'-nucleotidase activity. Another peak which coincides with that for G-6-Pase was located at a density of 1.2. Thus the distribution of the 'non-soluble' activity for UDPase is seen to be intermediate between that of AMPase and that of G-6-Pase. A rather similar distribution pattern was observed for IDPase.

A comparison of Table 2 with Table 1 reveals that the distribution of the 'non-soluble' ADPase activity (as distinct from UDPase and IDPase) and of Mg++-activated ATPase closely parallels that of AMPase. However, in the 'soluble' region
### Distribution of Nucleoside Phosphatases amongst Zonal Fractions (cf. Table 1)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>UDPase</th>
<th>IDPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run No:</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>% Zonal Activity*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D_{20} &lt; 1.10$</td>
<td>98%</td>
<td>87.5%</td>
</tr>
<tr>
<td>$1.10 &lt; D_{20} &lt; 1.17$</td>
<td>2%</td>
<td>10%</td>
</tr>
<tr>
<td>$D_{20} &gt; 1.17$</td>
<td>0%</td>
<td>2.5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ADPase</th>
</tr>
</thead>
<tbody>
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<td>Run No:</td>
<td>31</td>
</tr>
<tr>
<td>% Zonal Activity*</td>
<td></td>
</tr>
<tr>
<td>$D_{20} &lt; 1.10$</td>
<td>65%</td>
</tr>
<tr>
<td>$1.10 &lt; D_{20} &lt; 1.17$</td>
<td>28%</td>
</tr>
<tr>
<td>$D_{20} &gt; 1.17$</td>
<td>6.5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ATPase</th>
<th>UTPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run No:</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>% Zonal Activity*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D_{20} &lt; 1.10$</td>
<td>52%</td>
<td>6.5%</td>
</tr>
<tr>
<td>$1.10 &lt; D_{20} &lt; 1.17$</td>
<td>40%</td>
<td>65%</td>
</tr>
<tr>
<td>$D_{20} &gt; 1.17$</td>
<td>7.5%</td>
<td>28.5%</td>
</tr>
</tbody>
</table>

* Total activity of fractions recovered from the zonal rotor taken as 100%

% Recoveries typically found for enzyme activities loaded into rotor:

- ADPase, 60%; UDPase, 70%; IDPase, 85%; ATPase, 160%; UTPase, 78%.

† DOC Present
there is an increased activity for ADPase and a decreased activity for ATPase when compared with the activity for AMPase in the same region. This distribution pattern for nucleoside di- and triphosphatases, which is in agreement with the observations of Goldfischer et al. (1964) is illustrated in Figs. 7, 11 and 12. Comment will be made in the Discussion on the high recovery found for ATPase.

6(b) Relative Specific Activities of Phosphatases of fractions recovered from a 'B-IV' rotor after zonal centrifugation of 'microsomal material' (post-lysosomal supernatant) in the presence of Mg$$^{++}$$.

In some zonal runs protein values were determined for all fractions recovered from the rotor. The gradient was divided into four regions, with respect to density, corresponding to a 'soluble' region, a 'smooth' region (consisting mainly of smooth-surfaced membranes when examined under the electron microscope), an 'intermediate' region (containing both smooth-surfaced and rough-surfaced membranes) and a 'rough' region consisting mainly of rough-surfaced membranes (cf. Fig. 8).

The relative specific activities of phosphatases of these regions are outlined in Table 2b. This is another way of presenting the data previously given in Tables 1 and 2a. The preparation and zonal centrifugation conditions for run 30 were similar to those for run 29 (see Table 1) and in all cases both the homogenization medium and the gradient contained Mg$$^{++}$$ (to 0.005 M). In run 31 the homogenization medium was 0.14 M NaCl [see Section 2(d) of Chapter III]. Enzyme activities mentioned in this section refer to specific activities.
As can be seen from Table 2b the greatest enrichment in AMPase activity was found in the 'smooth' region. In this region a four-fold purification of the membrane fragments possessing AMPase activity was attained. Nevertheless considerable activity was also found in the 'intermediate' region.

On the other hand the greatest enrichment in G-6-Pase activity was found in the 'rough' region; purification of the G-6-Pase-containing membrane fragments in this region being almost four-fold. Considerable activity was also found in the 'intermediate' region. The activities for AMPase and for G-6-Pase in the 'intermediate' region are indicative of overlapping densities for the membranes which possess these enzyme activities i.e., mainly plasma membrane fragments (AMPase-containing fragments) and smooth-surfaced ER fragments (G-6-Pase-containing fragments).

ATPase (Mg\(^{++}\)-activated) activity was greatest in the 'smooth' and in the 'intermediate' regions [cf. Section 6(a) of this Chapter]. Activity in the latter region may be due to mitochondrial membranes contaminating the region. Although an electron microscopic examination did not reveal the presence of intact mitochondria, the possibility of mitochondrial fragments being present cannot be ruled out.

The specific activities of diphosphates in the zonal regions parallel closely the activities mentioned in Section 6(a) of this Chapter. The greatest enrichment in UDPase activity was found in the 'soluble' region (Table 2b).
This may be attributed to UDPase being readily solubilized or to its intracellular localization differing from that of IDPase and ADPase as shown cytochemically (Novikoff, Essner, Goldfischer and Heus, 1962; El-Aaser, 1965).

Reasonable reproducibility of relative specific activities of phosphatases was achieved in all regions except in the 'intermediate' region. Since the amount of protein recovered in this region was exceedingly small, this lack of reproducibility is hardly surprising. The activities of phosphatases in the four regions mentioned above will be given further consideration in the Discussion.
Phosphatase enrichment, relative to 'microsomal material' (post-lysosomal supernatant) of fractions recovered from a 'B-IV' zonal rotor (cf. Tables 1 and 2a). In all runs Mg** was present (0.005M).

<table>
<thead>
<tr>
<th>Region of gradient</th>
<th>Run No.</th>
<th>AMPase</th>
<th>G-6-Pase</th>
<th>ATPase</th>
<th>IDPase</th>
<th>UDPase</th>
<th>ADPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Soluble' (D20 &lt; 1.10)</td>
<td>27</td>
<td>0.22</td>
<td>0.12</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>0.18</td>
<td>0.11</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.23</td>
<td>0.13</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>31†</td>
<td>0.38</td>
<td>0.19</td>
<td>0.12</td>
<td>0.54</td>
<td>1.30</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>0.32</td>
<td>0.05</td>
<td>0.07</td>
<td>0.26</td>
<td>1.17</td>
<td>0.74</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.24(4)</td>
<td>0.10(4)</td>
<td>0.09(4)</td>
<td>0.26(1)</td>
<td>1.17(1)</td>
<td>0.74(1)</td>
</tr>
<tr>
<td>'Smooth' (1.10 &lt; D20 &lt; 1.16)</td>
<td>27</td>
<td>3.85</td>
<td>0.69</td>
<td>4.00</td>
<td></td>
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<tr>
<td></td>
<td>29</td>
<td>5.30</td>
<td>1.70</td>
<td>2.93</td>
<td></td>
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<tr>
<td></td>
<td>30</td>
<td>4.40</td>
<td>0.99</td>
<td>3.10</td>
<td></td>
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<tr>
<td></td>
<td>31†</td>
<td>3.95</td>
<td>0.98</td>
<td>4.15</td>
<td>1.42</td>
<td>0.50</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>2.46</td>
<td>0.48</td>
<td>2.80</td>
<td>1.36</td>
<td>0.49</td>
<td>2.12</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>4.00(4)</td>
<td>0.96(4)</td>
<td>3.21(4)</td>
<td>1.36(1)</td>
<td>0.49(1)</td>
<td>2.12(1)</td>
</tr>
<tr>
<td>'Intermediate' (1.16 &lt; D20 &lt; 1.18)</td>
<td>27</td>
<td>2.95</td>
<td>2.43</td>
<td>3.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>4.20</td>
<td>6.30</td>
<td>7.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.10</td>
<td>2.36</td>
<td>3.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>31†</td>
<td>1.18</td>
<td>1.62</td>
<td>1.48</td>
<td>1.57</td>
<td>0.54</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>2.54</td>
<td>1.44</td>
<td>2.93</td>
<td>2.10</td>
<td>0.60</td>
<td>0.86</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.95(4)</td>
<td>3.13(4)</td>
<td>4.20(4)</td>
<td>2.10(1)</td>
<td>0.60(1)</td>
<td>0.86(1)</td>
</tr>
<tr>
<td>'Rough' (D20 &gt; 1.18)</td>
<td>27</td>
<td>1.23</td>
<td>5.15</td>
<td>1.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>1.25</td>
<td>3.20</td>
<td>1.56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.43</td>
<td>4.05</td>
<td>2.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>31†</td>
<td>1.28</td>
<td>4.77</td>
<td>2.20</td>
<td>2.58</td>
<td>0.34</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>1.46</td>
<td>3.26</td>
<td>1.72</td>
<td>2.23</td>
<td>0.95</td>
<td>1.06</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>1.34(4)</td>
<td>3.91(4)</td>
<td>1.74(4)</td>
<td>2.23(1)</td>
<td>0.95(1)</td>
<td>1.06(1)</td>
</tr>
</tbody>
</table>

* Specific activity of region. / Specific activity of total fractions recovered from zonal rotor.
† Same enzyme as AMPase.
‡ Homogenization medium was 0.14 M NaCl.
Values in () refer to the number of experiments.
The average recovery of protein from the gradient was 80%.
For recovery values for enzymes see Tables 1 and 2a.
Sections from an ethionine-induced transplanted hepatoma were examined under the electron microscope in order to compare morphological details in various generations of transplantation. Fig. 17 (courtesy of M.S.C. Birbeck) is an electron micrograph of a section from a hepatoma after 9 generations of transplantation. The nuclei appear enlarged and the mitochondria, whose internal membrane system seems to be less orderly, are more rounded than in normal liver (see Fig. 1). In the hepatoma, ER membranes which in sections from normal liver are arranged in parallel arrays are lacking, at least in this highly organized form.

Fig. 18 is an electron micrograph of a section from a hepatoma after 44 generations of transplantation. Although the electron micrograph is of a higher magnification than for Fig. 17, and thus shows a smaller area of the section, no marked differences in ultrastructure from those exhibited in earlier generations of transplantation are evident.

2. Marker enzymes

A cytochemical examination of sections from a transplanted hepatoma (in this case after 38 generations of transplantation) was carried out to furnish information on the intracellular localization of phosphatase enzymes and to compare these localizations with those observed in sections from normal liver.
Legends to Figs 17-22

**Fig. 17** Electron micrograph of ethionine-induced hepatoma, sub-line 'UB', after 9 generations of transplantation. Note enlarged nuclei and abundance of rounded mitochondria and lack of parallel arrays of ER membranes. Lead stained.
Magnification x 6,000

**Fig. 18** Electron micrograph of hepatoma, sub-line 'UA', after 44 generations of transplantation showing nucleus (N), mitochondria (M), plasma membrane (PM) which is not well defined and which indicates poor cell contact, and lipid inclusions (L). Lead stained.
Magnification x 12,000

**Fig. 19** 5'-Nucleotidase (5'-UMP as substrate) in hepatoma, sub-line 'UA', after 38 generations of transplantation showing activity in the PM surrounding what looks like a star cell, but not in M or in a red blood cell at top right. Incubation 25 minutes at 37°.
Electron micrograph x 24,000

**Fig. 20** G-6-Pase in hepatoma, sub-line 'UA', after 38 generations of transplantation. This may be a binucleate cell since no plasma membrane between the two nuclei could be delineated. Deposits on NE and ER. Incubation 40 minutes at 37°.
Electron micrograph x 18,000

**Fig. 21** (a) and (b)
ATPase in hepatoma, sub-line 'UA', after 38 generations of transplantation, showing deposits of lead phosphate on PM. No deposition on M or L or in N. Incubation 45 minutes at 37°.
Electron micrographs x 30,000
Legends to Figs. 17-22 (continued)

**Fig. 22** Electron micrographs of pellets from zonal fractions obtained by centrifuging hepatoma (sub-lines 'UA' and 'UB') microsomal material (see Fig. 23). Note the similarity in appearance of the three fractions.

(a) Fractions 14-16 showing smooth-surfaced sheets of membrane and vesicles and also free ribosomes. Lead stained.

Magnification x 30,000

(b) Fractions 18-19 showing smooth-surfaced membranes and ribosome-like particles. Lead stained.

Magnification x 30,000

(c) Fractions 30-32 consisting mainly of smooth-surfaced vesicles. Lead stained.

Magnification x 30,000
Fig. 19 illustrates 5'-nucleotidase activity (with 5'-UMP as substrate) in a hepatoma section. A positive reaction is seen in the plasma membrane but no activity is observed in mitochondria or in the red blood cell (upper right).

When a hepatoma section was incubated to demonstrate G-6-Pase activity a faint positive reaction on the nuclear envelope was observed (Fig. 20). Although the deposition of lead phosphate in the cytoplasm is somewhat diffuse, enzyme activity is detected on membranous elements (ER). This faintness in staining for G-6-Pase coincides with the low activity found for G-6-Pase when assayed biochemically in the ethionine-induced hepatoma. ATPase activity in a hepatoma section is illustrated in Fig. 21, where a positive reaction in the plasma membrane is observed.

These cytochemical observations on sections from an ethionine-induced transplanted hepatoma give no evidence that the intracellular localization of 5'-nucleotidase, ATPase and G-6-Pase differs from that found in normal liver.

3. **Zonal centrifugation of hepatoma microsomal material**

in a 'B IV' rotor

Fig. 23 shows the distribution pattern for phosphatase enzymes and for RNA amongst 'microsomal material' prepared from an ethionine-induced transplanted hepatoma (sub-lines 'UA' and 'UB') and centrifuged for 2.5 hours in a 'B-IV' rotor. The homogenization medium (0.25 M sucrose) and the gradient contained Mg++ (to 0.005 M) and were buffered with 0.005 M Tris at pH 7.4. G-6-Pase activity is reduced to one-fourth of that found in normal liver but its distribution amongst the zonal fractions is similar to that observed with normal liver (see Table 3).
Microsomal material from 8.5 g. tumour was loaded.
Distribution of phosphatases amongst zonal fractions from hepatoma microsomal material (Run No. TIM). Compare with Tables 1 and 2 for normal liver.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>AMPase</th>
<th>G-6-Pase</th>
<th>ADPase</th>
<th>IDPase</th>
<th>Alk-β-G-Pase</th>
</tr>
</thead>
<tbody>
<tr>
<td>% zonal activity*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D_{20} &lt; 1.10$</td>
<td>49.5%</td>
<td>44%</td>
<td>49%</td>
<td>53.5%</td>
<td>32%</td>
</tr>
<tr>
<td>$1.10 &lt; D_{20} &lt; 1.17$</td>
<td>40%</td>
<td>2%</td>
<td>32.5%</td>
<td>20%</td>
<td>48%</td>
</tr>
<tr>
<td>$D_{20} &gt; 1.17$</td>
<td>10.5%</td>
<td>54%</td>
<td>18.5%</td>
<td>26.5%</td>
<td>20%</td>
</tr>
</tbody>
</table>

* Total activity of fractions recovered from the zonal rotor is taken as 100%.

% Recoveries found for enzyme activities loaded into rotor:

AMPase, 93%; ADPase, 150%; IDPase, 124%.

Alkaline β-glycerophosphatase activity (Alk-β-G-Pase, assayed at pH 9.6) and ADPase activity parallel that of AMPase. Again as with normal liver, IDPase exhibits a distribution intermediate between that of AMPase and that of G-6-Pase. When the zonal fractions were pumped from the rotor with monitoring of absorption 280 μ, it was noted that the absorption peak at a density of about 1.2 was much reduced (not illustrated in Fig. 23) when compared to that found with microsomal material from normal liver. This indicates a reduction in the amount of rough-surfaced ER membranes in the hepatoma being studied.

Zonal centrifugation of microsomal material from normal liver, in the presence of Mg++ ions, gave one main peak of RNA in a region of density 1.2. Electron microscopical examination
of fractions from under this RNA peak revealed the presence of rough-surfaced membranes (ER). Unlike the pattern found for normal liver the distribution pattern for RNA in hepatoma 'microsomal material' (in the presence of Mg\textsuperscript{++}) shows a rather disperse spread of RNA peaks (Fig. 23). The peaks in the 'central' region of the gradient probably correspond to free ribosomes which, under the conditions employed, would still be sedimenting. Comment on the high recoveries for the diphosphatases is deferred to the Discussion.

Fig. 22 illustrates the morphology of various zonal fractions which were centrifuged to give pellets, sections of which were examined under the electron microscope. Fractions 14 to 16 [Fig. 22 (a)], fractions 18 and 19 [Fig. 22 (b)] and fractions 30 to 32 [Fig. 22 (c)] each consisted of sheets of membrane and vesicular material, all being of the smooth-surfaced type. The distribution pattern for RNA, illustrated in Fig. 23, may account for the small particles resembling ribosomes which were observed in all the fractions examined.

In other experiments a crude nuclear fraction and a crude microsomal fraction from a transplanted hepatoma were examined cytochemically, as will be considered later.
CHAPTER IV

Cytochemical Studies on Tissue Fractions

1. Effects of glutaraldehyde fixation on enzyme activities

(a) Purification of glutaraldehyde

Commercial glutaraldehyde, purchased as a solution in water, was purified (see Chapter II, Section 7) by repeated treatment with charcoal in accordance with the method of Anderson (1967). The spectral absorption curves for untreated commercial glutaraldehyde and for purified glutaraldehyde are shown in Fig. 24 (a) and (b). Purified glutaraldehyde exhibits an absorption maximum at a wavelength of 280 m\(\mu\) [Fig. 24(a)], whereas a second absorption at 235 m\(\mu\) is evident in the case of the untreated glutaraldehyde [Fig. 24(b)]. The absence of the absorption peak at 235 m\(\mu\), due to contaminating material, was taken as the criterion for the purity of glutaraldehyde (Anderson, 1967).

Nominally the commercial solution contained 25% glutaraldehyde. When estimated by a hydroxylamine titration method (see Anderson 1967) the percentages (w/v) of glutaraldehyde in the commercial and purified solutions were 23.5% and 14% respectively.

(b) Inhibition of enzymes by glutaraldehyde

The effects of fixation with glutaraldehyde, a fixative frequently used in cytochemical work, on 5'-nucleotidase (UMPase) and G-6-Pase is shown in Table 4. Different concentrations of glutaraldehyde, including a purified sample, were employed in the study, and all solutions were adjusted to pH 7.4 with 0.1 M sodium cacodylate. Liver which had been homogenized in 0.25 M sucrose, buffered with 0.1 M sodium cacodylate pH 7.4, but which had not been fixed in glutaraldehyde, was used as a control. The results
Spectral absorption curve for 1% commercial glutaraldehyde. Note second absorption maximum at 235 μ, probably due to impurities.
are presented as the percentage activity remaining after fixation with glutaraldehyde, the activity of the controls being taken as 100%.

As can be seen from Table 4, fixation with glutaraldehyde causes a very marked inhibition of enzyme activity which is more pronounced for 5'-nucleotidase (UMPase) than for G-6-Pase. When used at the same concentrations, purified glutaraldehyde is slightly less inhibitory to enzyme activity than is the untreated commercial glutaraldehyde.
Table 4
Inhibition of enzymes by glutaraldehyde

Commercial glutaraldehyde solution was normally used. The concentrations (w/v) refer to glutaraldehyde itself, not to the solution.

<table>
<thead>
<tr>
<th>Tissue fraction</th>
<th>Glutaraldehyde concentration</th>
<th>Activity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5'-Nucleotidase (UMPase)</td>
</tr>
<tr>
<td>15,000 g pellet*</td>
<td>10%</td>
<td>2.2% (2)</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>2.3% (2)</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>2.4% (2)</td>
</tr>
<tr>
<td></td>
<td>3% (purified)</td>
<td>2.9% (2)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>3.2% (2)</td>
</tr>
<tr>
<td>Mic. pellet*</td>
<td>10%</td>
<td>2% (2)</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>2.3% (2)</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>2.5% (2)</td>
</tr>
<tr>
<td></td>
<td>3% (purified)</td>
<td>3% (2)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>2.5% (2)</td>
</tr>
</tbody>
</table>

* See Chapter II, Section 3(c). The pellets were fixed for 2 hr. at 4°C in glutaraldehyde at the different concentrations indicated. The fixed pellets were washed several times in 0.25 M sucrose buffered with 0.1 M sodium cacodylate pH 7.4, resuspended in 0.25 M sucrose and aliquots assayed for UMPase and for G-6-Pase.

Values in () refer to the number of experiments.
2. Effects of lead ions on enzyme activities

Lead ions, which act as trapping agents for released phosphate in cytochemical assays for phosphatases, are known to inhibit enzyme activities (Novikoff et al., 1958; El-Aaser, 1965). Certain phosphatases (UMPase, ATPase and G-6-Pase) were assayed biochemically at 37° in the presence of lead ions. The results are shown in Table 5. The effects of lead ions at the concentrations used in cytochemical assays and also at lower concentrations were investigated. Phosphatase activities in cytochemical media lacking lead ions served as control values (100% activity).

As can be seen from Table 5, the degree of inhibition seems to be less with microsomal material (Mic.) than with the original homogenate (Hom.). Enzyme inhibition was greatest when the concentrations of lead were those of the conventional cytochemical assays; namely, 3.6 × 10^{-3}M for UMPase and ATPase and 4.4 × 10^{-3}M for G-6-Pase. Lowering the concentration of lead ions decreased the inhibitory effect and at very low concentrations an increase in activity (with respect to the control) was observed. However, such low concentrations of lead are known to be insufficient to trap all of the released phosphate and cause antefactual staining due to diffusion of lead phosphate (Novikoff et al., 1958, Reid et al., 1964).

In order to check whether there was any non-enzymatic hydrolysis of the substrates UMP, ATP and G-6-P by lead ions (Moses and Rosenthal, 1967, Novikoff, 1967), these substrates were incubated at 37° for 15 minutes in the presence of lead ions (at the four different concentrations shown in Table 5) but in the absence of tissue. No release of phosphate could be detected.
Table 5

Effects of lead ions on enzyme activities

The lead salt added was lead nitrate.

<table>
<thead>
<tr>
<th>Tissue fraction</th>
<th>Lead concentration</th>
<th>Activity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5'-nucleotidase (UMPase)</td>
</tr>
<tr>
<td>Hom. *</td>
<td>$3.6 \times 10^{-3}$ M †</td>
<td>46% (3)</td>
</tr>
<tr>
<td></td>
<td>$3.6 \times 10^{-4}$ M</td>
<td>84% (3)</td>
</tr>
<tr>
<td></td>
<td>$1.8 \times 10^{-3}$ M</td>
<td>65% (3)</td>
</tr>
<tr>
<td></td>
<td>$1.8 \times 10^{-4}$ M</td>
<td>87% (3)</td>
</tr>
<tr>
<td>Mic. *</td>
<td>$3.6 \times 10^{-3}$ M †</td>
<td>61% (3)</td>
</tr>
<tr>
<td></td>
<td>$3.6 \times 10^{-4}$ M</td>
<td>115% (3)</td>
</tr>
<tr>
<td></td>
<td>$1.8 \times 10^{-3}$ M</td>
<td>93% (3)</td>
</tr>
<tr>
<td></td>
<td>$1.8 \times 10^{-4}$ M</td>
<td>116% (3)</td>
</tr>
<tr>
<td>Hom. *</td>
<td>$4.4 \times 10^{-3}$ M †</td>
<td>58% (2)</td>
</tr>
<tr>
<td></td>
<td>$4.4 \times 10^{-4}$ M</td>
<td>112% (2)</td>
</tr>
<tr>
<td>Mic. *</td>
<td>$4.4 \times 10^{-3}$ M †</td>
<td>52% (2)</td>
</tr>
<tr>
<td></td>
<td>$4.4 \times 10^{-4}$ M</td>
<td>113% (2)</td>
</tr>
</tbody>
</table>

* See Chapter II, Section 3 (c).

† Concentrations used in cytochemical assays throughout this study.

Values in ( ) refer to the number of experiments.
3. Cytochemical studies on a crude microsomal fraction

(a) Unfixed tissue

Thin sections of microsomal material prepared by method 'A' (see Section 8(b) of Chapter II), were cytochemically examined for the three enzyme activities, UMPase, ATPase and G-6-Pase. The pattern obtained for UMPase is shown in Fig. 25(a).

Deposits of lead phosphate are located on sheets of membrane and also on some vesicles. The pattern obtained for a section incubated for ATPase is shown in Fig. 25(b). Several small sheets of membrane and vesicles exhibit enzyme activity.

Sheets of membrane similar to those exhibiting activity for UMPase displayed no activity when a section was incubated for G-6-Pase [Fig. 25(c)]. Deposition of lead phosphate was observed on a tubular membranous element [bottom right in Fig. 25(c)] and on some vesicles presumably derived from the ER. Comment will be low made later on the proportion of vesicles exhibiting G-6-Pase activity in Fig. 25(c).

When a section was incubated in a medium similar to that employed to demonstrate UMPase activity but lacking the substrate UMP, no deposition of lead occurred [Fig. 25(d)]. However, an increase in the contrast of ribosomes was observed, possibly attributable to an uptake of lead from the control medium by these particles. In all cytochemical experiments reported in this thesis, no deposition of lead was observed in sections which were incubated as controls in media lacking substrates.

Quite a large number of membrane fragments have exhibited no activity for either of the three enzymes. This can be interpreted
as reflecting the heterogeneity encountered with membrane fragments isolated in the microsomal fraction and it is quite possible that fragments derived from cellular organelles, e.g. mitochondria and microbodies, are present in the fraction along with plasma membrane and ER fragments. Nevertheless the paucity of elements showing activity could be due to a defect in the cytochemical technique. Work entailing longer incubation times, as outlined later in this Chapter, is relevant in this connection.

Legend to Fig. 25

Cytochemistry on a crude, unfixed microsomal fraction [see Chapter II, Section 8(b)]. The microsomal pellet was homogenized in 1M sucrose and thin sections prepared. [Method 'A' (see Chapter II, Section 8(b)).]

(a) 5'-Nucleotidase, 5'-UMP as substrate. Deposits of lead phosphate localized on large sheets of membrane (SH) at top left and on vesicles (v) at bottom left.

Incubated at 18° for 5 minutes

Electron micrograph x 30,000

(b) ATPase. Activity on some small sheets of membrane (SH) and on vesicles (v). Many small vesicles gave negative reaction.

Incubated at 18° for 5 minutes

Electron micrograph x 30,000

(c) G-6-Pase. Deposition of lead phosphate on vesicles (v) at bottom left, and on tubular membrane fragment (bottom right). No deposition on sheet of membrane (SH) at right of centre.

Incubated at 18° for 5 minutes

Electron micrograph x 30,000
(d) Control. 'Section' incubated in a medium similar to that used in (a) above but lacking the substrate UMP. No deposition of lead. Note enhanced contrast of ribosomes. Incubated at 4°C for 5 minutes
Electron micrograph x 30,000

(b) Glutaraldehyde-fixed tissue

Fig. 26 shows the results obtained when a microsomal pellet was fixed in unpurified commercial glutaraldehyde (buffered with 0.1 M sodium cacodylate, pH 7.4) at 4°C for 1 hr., thoroughly washed in 0.25 M sucrose (buffered as above) and cytochemically examined. Thin sections of the fixed material were prepared by method 'A' [see Section 8(b) of Chapter II]. Fig. 26(a) is an electron micrograph of a section fixed in 3% glutaraldehyde and incubated for UMPase. Although good morphological preservation was not attained, it could be concluded that no deposition of lead phosphate had occurred. This indicates the marked inhibitory effect that the fixative has on the enzyme.

In further work the incubation time was lengthened and the concentration of glutaraldehyde was lowered. Fig. 26(b) is an electron micrograph of a thin section (prepared by method 'A') of microsomal material fixed in 1.5% commercial glutaraldehyde buffered with 0.1 M sodium cacodylate pH 7.4 and cytochemically examined for UMPase. A light deposition of lead phosphate, some of which is located on membrane fragments i.e. vesicles at centre of Fig. 26(b), can be observed.

Similar results (not illustrated) were obtained for ATPase and for G-6-Pase, i.e., no activity was observed with sections fixed in
3% glutaraldehyde but moderate activity was achieved with sections fixed in 1.5% glutaraldehyde. These cytochemical observations agree with those on the inhibition of phosphatase enzymes by glutaraldehyde as outlined in Table 4. Further observations on tissue fractions fixed in purified glutaraldehyde will be considered later.

Legend to Fig. 26

Cytochemistry on a crude microsomal fraction fixed in commercial glutaraldehyde. After fixation the microsomal pellet was homogenized in 0.25 M sucrose and thin sections were prepared. [Method 'A', see Chapter II, Section 8 (b)].

(a) 5'-Nucleotidase, 5'-UMP as substrate in a microsomal fraction fixed in 3% glutaraldehyde for 1 hr. at 4°.

Due to the inhibitory effect of the glutaraldehyde no deposition of lead phosphate was observed.

Incubated at 18° for 15 minutes

Electron micrograph x 30,000

(b) 5'-Nucleotidase, 5'-UMP as substrate, in a microsomal fraction fixed in 1.5% glutaraldehyde for 1 hr. at 4°.

Deposits of lead phosphate were located on some vesicles (v) at centre.

Incubated at 18° for 90 minutes

Electron micrograph x 30,000

(c) Variation of incubation time

The aim was to obtain evidence on the specificity of 'staining' in a population of microsomal vesicles. Sections 30μ thick were cut from a frozen unfixed microsomal pellet [method 'B', see Chapter II, Section 8(b)] and incubated in media to demonstrate 5'-nucleotidase
UMPase and G-6-Pase activities. The time of incubation was varied between 5 minutes and 2 hours (1 hour in the case of G-6-Pase) in order to observe whether an increase in time brought about an increase in the proportion of membrane fragments exhibiting enzyme activity.

Fig. 27(a) is an electron micrograph of a section incubated for UMPase for a period of 5 minutes. Deposition of lead phosphate occurred on a few vesicles. UMPase activity in a section incubated for 30 minutes [Fig. 27(b)] revealed no marked difference in its staining pattern when compared with that observed after 5 minutes incubation. An increase in the intensity of the staining reaction was noted with an incubation time of 2 hours, but good morphological preservation was not attained [Fig. 27(c)]. The proportion of fragments showing deposits was little changed (about one half).

Fig. 28(a to d) illustrates the pattern observed when sections (similar to those mentioned above) were incubated for varying periods of time in a medium to demonstrate G-6-Pase activity. With incubation for 5 minutes only a few vesicles exhibited definite activity, as judged by an intense staining reaction [top left of Fig. 28(a)]. Nevertheless a faint deposition of lead phosphate was noted on many vesicles. The intensity of the deposition increased after 15 minutes incubation [Fig. 28(b)], and after 30 minutes the majority of the vesicles exhibited activity [Fig. 28(c)]. Incubation for 1 hour [Fig. 28(d)] resulted in an increase in the intensity of the staining reaction, comparable with that observed in a section incubated for UMPase for 2 hours [see Fig. 27(c)]. The proportion of vesicles stained was hardly changed.

No deposition of lead was observed when a section was incubated for 1 hour in a medium similar to that used to demonstrate G-6-Pase activity but lacking in the substrate G-6-P [Fig. 28(e)]. However, the contrast of ribosomes was enhanced, this possibility being
attributable to an uptake of lead from the medium by these particles [see Fig. 25(d)].

It should be stressed that when the incubation time was 5 minutes, few vesicles exhibited G-6-Pase activity whereas many exhibited UMPase activity [see Figs. 27(a) and 28(a)]. This paucity of vesicles exhibiting G-6-Pase activity may be due to a lag period with G-6-Pase.

Most of the G-6-Pase activity and less than half of the 5'-nucleotidase activity (assayed biochemically) of a liver homogenate was recovered in the microsomal fraction, under the conditions employed in experiments reported here. Hence in the microsomal fraction a greater proportion of membrane fragments would be expected to exhibit activity for G-6-Pase than for 5'-nucleotidase. That such is the case can be seen when a section was incubated for G-6-Pase for 30 minutes [Fig. 28(c)]. Here most of the membrane fragments gave a positive reaction [compare with Fig. 27(b)].

Studies in progress in this laboratory at the present time are concerned with the improvement of techniques employed in the cytochemical examination of the microsomal fraction. Fixation in glutaraldehyde for a short period of time (5 minutes) and the use of a microsomal suspension rather than a pellet may be beneficial. Such conditions might reduce artifacts caused by diffusion of lead phosphate and by the different rates of penetration of substrates, such as can occur with an unfixed pellet.
Legend to Fig. 27

5′-Nucleotidase, 5′-UMP as substrate, in a crude unfixed microsomal fraction. Thin sections from the microsomal pellet were prepared by method 'B' [see Chapter II, Section 8(b)] and incubated for varying periods of time.

(a) Incubated at 18° for 5 minutes. Deposition of lead phosphate on vesicles (v) and on small sheets of membrane (SH). No activity on many small vesicles presumably derived from the ER.

Electron micrograph x 30,000

(b) Incubated at 18° for 30 minutes. Deposition of lead phosphate on vesicles (v). Staining reaction similar to that observed in (a) above.

Electron micrograph x 30,000

(c) Incubated at 18° for 2 hr. Deposits of lead phosphate were localized on vesicles (v) similar in appearance to those exhibiting activity in (a) and (b) above. Note increase in the intensity of the staining reaction. Good morphological preservation of membranes was not attained.

Electron micrograph x 30,000
Legend to Fig. 28 (a to d)

G-6-Pase in a crude unfixed microsomal fraction. Thin sections [method 'B' see Section 8 (b) of Chapter II] were incubated for varying periods of time.

(a) Incubated at 18° for 5 minutes. Deposition of lead phosphate on vesicles (v) at top left and bottom right. Note light deposits on majority of vesicles.
   Electron micrograph x 30,000

(b) Incubated at 18° for 15 minutes. Deposits of lead phosphate localized on many vesicles (v). Intensity of staining reaction greater than that in (a) above.
   Electron micrograph x 30,000

(c) Incubated at 18° for 30 minutes. Majority of vesicles (v) gave a positive reaction. Some deterioration in morphology of vesicles noted.
   Electron micrograph x 30,000

(d) Incubated at 18° for 1 hr. Pattern similar to that observed in (c) above except that intensity of staining reaction increased.
   Electron micrograph x 30,000

(e) Control. Section incubated at 18° for 1 hr. in a medium similar to that used in (a) to (d) above but lacking the substrate G-6-P. No deposition of lead was observed.
   Note enhanced contrast of ribosomes.
   Electron micrograph x 30,000
4. Cytochemical observations on tissue fractions isolated by zonal centrifugation

(a) Fractions obtained by zonal centrifugation of a crude nuclear fraction in an 'A' rotor

A plasma membrane fraction was isolated by zonal centrifugation of a crude nuclear fraction from liver in an 'A' rotor (El-Aaser et al., 1966a). Electron microscopic examination of this plasma membrane fraction, which was enriched in 5'-nucleotidase activity (assayed biochemically), revealed many large irregular sheets of membrane similar to those isolated by Emmelot et al. (1964b) and also some vesicles [Fig. 29(a)]. Zonal centrifugation of a crude nuclear fraction in an 'A' rotor yielded, in addition to a plasma membrane fraction, one rich in succinic dehydrogenase (assayed biochemically) and which consisted of mitochondria, many of which were swollen [Fig. 29(b)].

Fig. 29(c) is an electron micrograph showing small sheets of membrane and vesicles, both rough-surfaced and smooth-surfaced, which sediment slowly upon zonal centrifugation of the crude nuclear fraction (in the 'A' rotor). The smooth-surfaced membranes represent plasma membrane fragments admixed with ER fragments. This was shown by recentrifuging the 'slowly sedimenting fractions' (see above) in a high speed 'B' zonal rotor, the 5'-nucleotidase being located in a region of the gradient free from G-6-Pase activity and from RNA. The small membrane fragments shown in Fig. 29(c) are similar in appearance to fragments isolated from microsomal preparations [see Fig. 8(d) in Chapter III].
In general, electron micrographs of the mitochondrial region show many of the mitochondria to be swollen and lacking in internal structure. These mitochondrial ghosts are sometimes seen in electron micrographs to associate with plasma membranes as a contaminant. It may therefore be difficult to distinguish between these ghosts and sheets of plasma membrane. A cytochemical examination of zonal fractions was carried out in order to help in the identification of plasma membrane fragments and to distinguish these from cytomembrane and other membrane fragments.

Legend to Fig. 29

Electron micrographs of various fractions obtained by zonal centrifugation of a crude nuclear fraction in an 'A' rotor.

(a) Plasma membrane fraction, containing some vesicles (v) at bottom left, and many large irregular sheets of membrane(Sh). Lead stained.

Magnification x 24,000

(b) Mitochondrial fraction. Some of the mitochondria are swollen and lack internal structures (cristae). Lysosomes (L) are sometimes detected in this fraction. Lead stained.

Magnification x 16,000

(c) Slowly sedimenting fraction consisting mainly of small vesicles (v) both rough-surfaced and smooth-surfaced. Lead stained.

Magnification x 18,000
Consideration will now be given to the cytochemical examination of membrane fractions obtained by zonal centrifugation of a crude nuclear fraction. In some cases the 'zonal' plasma membrane fraction was further purified by recentrifugation in discontinuous sucrose gradients (Hinton, 1969).

In the experiment now being considered, a zonal fraction possessing both 5'-nucleotidase and G-6-Pase activities (assayed biochemically), and thus was not a pure plasma membrane fraction, was cytochemically examined for the same two enzymes. The aim of separating plasma membrane fragments (5'-nucleotidase-containing fragments) from those of other membranes was not achieved and the fraction was contaminated with nuclei. Thin 'sections' from the unfixed zonal fraction were prepared by Method 1A. Fig. 30(a) is an electron micrograph of one such section incubated for UMPase. Deposits of lead phosphate were localized on sheets of membrane probably derived from the plasma membrane. No such deposition occurred on structures resembling swollen mitochondria (top left) or on fragments of membrane presumably derived from the ER.

The result of incubating another thin 'section' from the same zonal fraction for G-6-Pase is shown in Fig. 30(b). Deposits of lead phosphate can be seen on the nuclear envelope, on vesicles and on membranes, arranged in parallel arrays which bear resemblance to ER membranes, as seen in electron micrographs of liver sections (see Fig. 3).
Inadequate fixation may account for the poor morphological preservation of nuclei as seen in Fig. 30(b). Sheets resembling those presumably derived from the plasma membrane, as illustrated in Fig. 30(a), could not be distinguished in Fig. 30(b).

Legend to Fig. 30

Cytochemistry on a fraction obtained by zonal centrifugation of a crude nuclear fraction in an 'A' rotor. Biochemical assays for 5'-nucleotidase and for G-6-Pase revealed high activities for these two enzymes in the fraction indicative of poor separation of the different elements (see text). The zonal fraction was centrifuged yielding a pellet which was resuspended in 0.25 M sucrose by agitation on a 'whirlimix'. Thin sections were prepared by method 'A' (see Section 8(b) of Chapter II).

(a) 5'-Nucleotidase, 5'-UMP as substrate. Light deposition of lead phosphate on sheets presumed to be plasma membrane (PM). No deposition on membranes presumably derived from ER or from swollen mitochondria (M).

Incubated at 18⁰ for 15 minutes.

Electron micrograph x 30,000

(b) G-6-Pase. Lead phosphate deposits on the nuclear envelope (NE) and on ER membranes.

Incubated at 18⁰ for 25 minutes

Electron micrograph x 12,000
A zonal fraction having considerable 5'-nucleotidase activity and lacking G-6-Pase and succinic dehydrogenase activities (all three enzymes having been assayed biochemically, the latter enzyme being a 'marker' for mitochondria) was cytochemically examined (method 'C', the thin pellet being unfixed) to demonstrate 5'-nucleotidase activity. This zonal fraction, unlike that considered previously, is a relatively pure plasma membrane fraction. Deposition of lead phosphate occurred on structures resembling bile canaliculi and on sheets of membrane presumably derived from the plasma membrane [Fig. 31(a) and (b)]. Yet many such sheets showed no activity, an observation which will be discussed later. No deposits were observed within or on the surface of mitochondria or in lysosomes [Fig. 31(b)], indicating the lack of non-specific deposition or adsorption of lead phosphate on to these cellular organelles.

An unfixed 'zonal' plasma membrane fraction, similar to that employed for the demonstration of 5'-nucleotidase activity (see above), was incubated for G-6-Pase. The results are shown in Fig. 32(a) and (b). Bile canaliculi and sheets of membrane, similar to those which exhibited activity for 5'-nucleotidase, did not reveal any activity for G-6-Pase. However, deposits of lead phosphate were localized on small vesicles which were in the vicinity of, and in some cases adhering to, the plasma membrane [Fig. 32(a) and (b)]. This may account for the residual G-6-Pase activity which is often found in plasma membrane preparations isolated from the nuclear fraction. Such activity is in all probability due to vesicles derived from the ER, which possess G-6-Pase activity and which, by adhering to the plasma membrane, contaminate the preparation.
Legends to Figs. 31 and 32

Cytochemistry on a plasma membrane fraction [see Fig. 29(a)] isolated by zonal centrifugation of a crude nuclear fraction in an 'A' rotor. The fraction possessed high 5'-nucleotidase activity and little or no G-6-Pase activity. Thin unfixed pellets were incubated [Method 'C'; see Chapter II, Section 8(b)].

Fig. 31
(a) 5'-Nucleotidase, 5'-UMP as substrate. Activity observed on some sheets of membrane (SH) and on structures resembling bile canaliculi (BC).

Incubated at 18° for 5 minutes
Electron micrograph x 18,000

(b) 5'-Nucleotidase as in (a) above. Activity on sheets of membrane (SH) but not in mitochondria (M) or lysosomes (L).

Incubated at 18° for 5 minutes
Electron micrograph x 18,000

Fig. 32
(a) and (b) G-6-Pase. Deposition of lead phosphate on small vesicles (v) adhering to the plasma membrane. No activity on bile canaliculi (BC) or on sheets (SH) probably derived from PM.

Incubated at 18° for 30 minutes
Electron micrograph x 30,000
A zonal plasma membrane fraction was further purified by recentrifugation in a swing-out rotor. This method yielded a purified fraction with considerable 5'-nucleotidase activity (the specific activity of UMPase being raised in this fraction) and low succinic dehydrogenase, and also a second fraction which possessed both activities. Fig. 33(a) illustrates 5'-nucleotidase in such a purified plasma membrane fraction [method 'C'; Chapter II, Section 8(b)]. Lead phosphate deposits were located on the majority of the membrane fragments, both sheets and vesicles. A difference in the intensity of the staining reaction was noted within the pellet. This may be attributed to a difference in the rate of penetration of substrate into such a pellet. When the second fraction (see above) was incubated for 5'-nucleotidase, deposition of lead phosphate occurred on fragments of membrane presumably derived from the plasma membrane, and on structures resembling damaged bile canaliculi [Fig. 33(b)]. Membrane fragments lacking 5'-nucleotidase activity, i.e., those not exhibiting lead phosphate deposition as illustrated in Fig. 33(b), are probably derived from damaged mitochondria or from other cellular organelles.

Since activity for G-6-Pase (assayed biochemically) was extremely low in both fractions, a cytochemical examination for this enzyme was not carried out (see Fig. 32).
Legend to Fig. 33

Cytochemistry on a plasma membrane fraction [cf. Fig. 29(a)] isolated by zonal centrifugation of a crude nuclear fraction in an 'A' rotor. The zonal fraction was further purified by recentrifugation on a discontinuous sucrose gradient. This step yielded a fraction (termed PM') possessing high activity for 5'-nucleotidase but lacking succinic dehydrogenase activity and a second fraction (termed PM") possessing both activities (see text). Thin unfixed pellets were incubated [method 'C'; Chapter II, Section 8(b)].

(a) 5'-Nucleotidase, 5'-UMP as substrate, in PM'.
Almost all membrane fragments, both sheets (SH) and vesicles (v), gave a positive reaction.
Note greater intensity of staining reaction at right of centre than at left.
    Incubated at 18° for 5 minutes
    Electron micrograph x 18,000

(b) 5'-Nucleotidase, 5'-UMP as substrate, in PM".
Activity on sheets of membrane (SH) and on a damaged bile canaliculus (BC). Membranes lacking deposits may have originated from damaged mitochondria.
    Incubated at 18° for 5 minutes
    Electron micrograph x 18,000
In order to ascertain whether improved enzyme localization could be achieved by fixation, a zonal plasma membrane fraction prepared from a crude nuclear fraction and containing some G-6-Pase, was centrifuged and the pellet fixed, for two hours at 4°, in 3% purified glutaraldehyde buffered with 0.1 M dimethylglutarate pH 7.4 (see Section 1(a) of this Chapter and also Table 4). The fixed thin pellet was washed for two hours in 0.25 M sucrose, buffered as above, and incubated in media to demonstrate cytochemical activities for 5'-nucleotidase and for G-6-Pase (method 'C'). 5'-Nucleotidase activity in such a fixed plasma membrane preparation is illustrated in Fig. 34(a). Deposits of lead phosphate were localized on some sheets of membrane, suggesting their plasma membrane origin. This result with purified glutaraldehyde is in contrast to the lack of activity noted for 5'-nucleotidase in a microsomal pellet fixed in 3% commercial glutaraldehyde [see Fig. 26(a)].

Fig. 34 (b) is an electron micrograph of a plasma membrane fraction (similar to that mentioned above) fixed in 3% purified glutaraldehyde and incubated for G-6-Pase. Deposition of lead phosphate occurred on small vesicles and on tubular membranous elements similar in appearance to those isolated from microsomal material [see Chapter III, Fig. 8(d)]. The longer incubation time for G-6-Pase than for UMPase may account for the higher proportion of membrane fragments exhibiting G-6-Pase activity [Fig. 34(b)] as compared to that exhibiting UMPase activity [Fig. 34(a)].
Fixation with glutaraldehyde, commercial or purified, inhibits greatly the activities of 5'-nucleotidase and G-6-Pase (see Table 4). This may account for the large number of membrane fragments which show no activity for either of these enzymes when fixed membrane preparations are cytochemically examined. Nevertheless more acceptable cytochemical results are evidently obtainable with tissue fractions fixed in purified glutaraldehyde, even with a prolonged fixation time and shorter incubation times.

Legend to Fig. 34

Cytochemistry on a zonal fraction [cf. Fig. 29(a)] isolated by zonal centrifugation of a crude nuclear fraction in an 'A' rotor. The zonal fraction (essentially a plasma membrane fraction but possessing some G-6-Pase activity) was centrifuged and the pellet fixed for 2 hr. at 4° in 3% purified glutaraldehyde [see Section 1(a) of this Chapter]. The thin pellet was incubated in media to demonstrate 5'-nucleotidase and G-6-Pase activities [method 'C'; see Section 8(b) of Chapter II].

(a) 5'-Nucleotidase, 5', UMP as substrate. Light deposition of lead phosphate occurred on some sheets (SH) of membrane (top centre). Compare with results obtained for unfixed zonal fractions (cf. Figs. 31 and 33).

Incubated at 18° for 10 minutes

Electron micrograph x 30,000

(b) G-6-Pase. Activity on membrane fragments, both tubular and vesicular (v), presumably derived from ER. No activity on sheets (SH) at right of centre.

Incubated at 18° for 40 minutes

Electron micrograph x 30,000
Fractions obtained by zonal centrifugation of microsomal material in a high speed 'B' rotor

5'-Nucleotidase-containing membrane fragments are known to sediment with the microsomal fraction as well as with the nuclear fraction. Success has been achieved in the separation of these plasma membrane fragments from membrane fragments derived from the ER by using high speed zonal rotors (see Chapter III, Section 2). Separations as effective as those reported for the 'B-IV' rotor in the previous chapter have been accomplished by using a 'B-XV' rotor (Norris, K.A., unpublished experiments). To offset the relatively low speed of the 'B-XV' rotor the time of centrifugation had to be increased.

A zonal fraction (obtained by zonal centrifugation of microsomal material' in a 'B-XV' rotor), possessing considerable 5'-nucleotidase activity but lacking a G-6-Pase activity in biochemical assays was cytochemically examined for 5'-nucleotidase (method 'C'). Sheets of membrane and vesicles exhibited activity, as can be seen from Fig. 35(a) and (b). Since no G-6-Pase activity was biochemically detected in this fraction and since only a limited amount of membranous material was available, a cytochemical examination for G-6-Pase was not carried out. Many small vesicles in Fig. 35 gave a negative reaction. Such vesicles may have originated as the result of fragmentation of the ER or of the outer mitochondrial membrane. In line with this latter point monoamine oxidase activity, a 'marker' enzyme for the outer mitochondrial membrane (Schnaitman, Erwin and Greenwalt, 1967), was detected in the fraction.

When a zonal fraction known to possess 5'-nucleotidase and G-6-Pase activities (the ratio of activities being 1:5 to 1) in
biochemical assays was incubated for 5'-nucleotidase, the results obtained are shown in Fig. 36. Deposits of lead phosphate were localized on small sheets of membrane and on vesicles. These 5'-nucleotidase-containing fragments might well be derived from the microvilli of the blood sinusoidal wall and of the bile canaliculi caused by the disruption of such microvilli during homogenization.

Fig. 37 is an electron micrograph of a similar zonal fraction incubated for G-6-Pase. Many vesicles of varying size exhibited enzyme activity. Monoamine oxidase activity was detected in this zonal fraction and may account for the many vesicles which have neither 5'-nucleotidase nor G-6-Pase activities.

Legend to Figs. 35 - 37

Cytochemistry on fractions obtained by zonal centrifugation of 'microsomal material' in a 'B-XV' rotor (see text). The zonal fraction examined in Fig. 35 corresponds to that obtained in tubes 16-20 of run 32, a 'B-IV' run, while the fraction examined in Figs. 36 and 37 corresponds to that obtained in tubes 21-27 of the same run (see Figs. 7 and 8). The fractions were not fixed and thin pellets were incubated [method 'C', see Section 8(b) of Chapter II].

Fig. 35 (a) and (b) 5'-Nucleotidase, 5'-UMP as substrate, in a fraction known to possess high 5'-nucleotidase activity and low G-6-Pase activity (assayed biochemically). Sheets of membrane (SH) gave a positive reaction. Two vesicles (v) in centre (top and bottom) of (b) exhibit activity.
Incubated at 18° for 10 minutes
Electron micrographs x 30,000

Fig. 36 5'-Nucleotidase, 5'-UMP as substrate, in a fraction possessing 5'-nucleotidase and G-6-Pase activities in biochemical assays. Deposits of lead phosphate were localized on small sheets of membrane (SH) and on vesicles (v) presumably derived from the plasma membrane.

Incubated at 18° for 10 minutes
Electron micrograph x 30,000

Fig. 37 G-6-Pase in a fraction similar to that employed in Fig. 36. Many vesicles (v) but no sheets gave a positive reaction. A large vesicle overlapping two smaller ones, only the former giving a positive reaction was observed in the centre of the micrograph.

Incubated at 18° for 20 minutes
Electron micrograph x 30,000
Cytochemical studies on tissue fractions isolated from a transplanted hepatoma

An ethionine-induced hepatoma, subline 'UB' after 71 generations of transplantation, was homogenized in 0.25 M sucrose and filtered through a sieve of fine mesh to remove fibrous tissue. From this homogenate a 15,000 g pellet and, subsequently, a microsomal pellet were prepared [see Chapter II, Section 3(c)]. Thin sections from these unfixed pellets were prepared (method 'B') and incubated in media to demonstrate cytochemically, 5'-nucleotidase and G-6-Pase activities.

The localization of lead phosphate deposits were somewhat ill-defined when a section from a 15,000 g pellet was incubated for 5'-nucleotidase (not illustrated). Nevertheless, some small sheets of membrane and also some vesicles did give a positive reaction.

The pattern obtained for a section of an unfixed 15,000 g pellet incubated for G-6-Pase is illustrated in Fig. 38(a). Deposition of lead phosphate on the nuclear envelope was observed. The level of G-6-Pase, assayed biochemically in the ethionine-induced hepatoma after 40 transplant generations was about one quarter of that found in normal liver, and this level has dropped even further with subsequent transplantation. This decrease in enzyme activity, as compared with normal liver, may account for the decrease in the proportion of hepatoma membrane fragments exhibiting G-6-Pase activity.
5'-nucleotidase activity in a section from an unfixed microsomal pellet is illustrated in Fig. 38 (b). Deposits of lead phosphate were observed on fragments of membrane presumably derived from the plasma membrane. A heavy deposit on a membrane fragment in the centre of the field can be seen while adjacent fragments lack such deposits.

Only a few small vesicles exhibited activity for G-6-Pase in a section from an unfixed microsomal pellet [Fig. 38(c)]. Again this is in accordance with the biochemical evidence for a decrease in G-6-Pase activity in the hepatoma.

Good morphological preservation of hepatoma tissue fractions, when compared with that found for tissue fractions from normal liver, was not achieved (compare Fig. 38 with Fig. 25). This may be attributed to the inadequate fixation of hepatoma membranes or to a difference in the chemical composition of these membranes (relative to normal liver) which may have affected their fixation properties. Further comment on the morphology of hepatoma tissue fractions will be made in the Discussion.

Legend to Fig. 38

Cytochemistry on a 15,000 g pellet and on a microsomal pellet (see text) prepared from a hepatoma (subline 'UB' after 71 generations of transplantation) homogenate. The pellets were not fixed prior to incubation and thin sections were prepared by method 'B' [see Section 8(b) of Chapter II].
(a) G-6-Pase in a section from a 15,000 g pellet. Deposition of lead phosphate occurred on the nuclear envelope (NE).

Incubated at 18° for 20 minutes

Electron micrograph x 30,000

(b) 5'-Nucleotidase, 5'-UMP as substrate, in a section from a microsomal pellet. Activity on small sheet of membrane (SH) in centre of micrograph and on many vesicles (v).

Incubated at 18° for 20 minutes

Electron micrograph x 30,000

(c) G-6-Pase in a section from a microsomal pellet. Only a few vesicles (v), at right of centre, gave a positive reaction. Poor morphological preservation was observed.

Incubated at 18° for 20 minutes

Electron micrograph x 30,000
CHAPTER V

Discussion

A. Biochemical work on normal liver.

1. Marker enzymes for biochemical work.

Much of the work reported in this thesis is concerned with the centrifugal isolation from liver homogenates of plasma membrane fragments free from ER fragments. Identification of the different membrane fragments is helped by examination of fractions for 'marker' enzymes corresponding to the different cellular elements. The cytochemical approach to the problem is to establish for tissues and tissue fractions a staining reaction for each enzyme that is assayed biochemically, and thereby find which of the elements present in tissue fractions contain the enzyme.

When a liver section which had been lightly fixed in glutaraldehyde was incubated for 5'-nucleotidase, deposition of lead phosphate occurred on the plasma membrane (Fig. 2), in agreement with the observations of Essner et al. (1958), Sabatini et al. (1963) and El-Aaser (1965). Heavy deposits were noted on the plasma membrane where it folds to form the bile canaliculus. This apparent increased enzyme activity is probably due to the increased surface area of the plasma membrane in the bile canalicular region rather than to higher specific activity in that region. Enzyme activity was also localized in the microvilli that form the blood sinusoidal wall.
The activity in the bile canaliculi and sinusoids suggests the involvement of 5'-nucleotidase in transport processes. No activity was observed in the ER membranes, mitochondria or on the nuclear envelope. Deposition of lead phosphate occurred in lysosomes (Fig. 2), this probably being due to the presence in these organelles of a non-specific acid phosphatase. El-Aaser (1965) concluded that lysosomes did not possess 5'-nucleotidase. This disagrees with the observations of Hardonk (1968) who stated that two 5'-nucleotidases exist in rat liver, one acting at a neutral pH and the other in an acid pH range. Hardonk considered the acid nucleotidase to be a lysosomal enzyme. In connection with the frequent use of 5'-UMP rather than 5'-AMP as substrate it should be noted that this hardly matters, since El-Aaser (1965) and Hardonk (1968) have shown that the same enzyme hydrolyses all the ribonucleoside monophosphates.

In confirmation of literature cited in the Introduction, the validity of using G-6-Pase as an ER marker has been established. Enzyme activity was located specifically in the ER membranes and on the nuclear envelope, the latter being continuous with the ER. The lead deposits obscure any ribosomes that are present on the ER. However, Orrenius and Ericsson (1966) were able to demonstrate G-6-Pase activity cytochemically on both smooth surfaced and rough surfaced ER in rat liver following phenobarbital treatment, which causes proliferation of the smooth surfaced ER membranes.
The localization of Mg\(^{++}\)-activated ATPase in a liver section was as illustrated in Fig. 4. As was mentioned in Chapter II the pH of the dimethylglutarate buffer was adjusted with NaOH so that additional activation by Na\(^+\) cannot be ruled out. The plasma membrane gave a positive reaction and activity was greatest in the bile canaliculus and sinusoidal wall, as was the case with 5′-nucleotidase. Activity was also demonstrated on a red blood cell [Fig. 4(a)].

In line with this latter observation Marchesi and Palade (1967) found staining for ATPase (both Mg\(^{++}\)-activated and Mg\(^{++}\)-Na\(^+\)-K\(^+\)-activated) on red cell ghost membranes.

No ATPase activity was demonstrated on the nuclear envelope or in ER membranes, either smooth surfaced or rough surfaced. The fact that no activity could be demonstrated cytochemically in mitochondria is a significant one, for subcellular fractionation studies on rat liver have shown the mitochondrial fraction to possess considerable ATPase activity in biochemical assays. Many authors were unable to demonstrate mitochondrial ATPase activity cytochemically, at least in rat liver sections, and attributed this inability to an adverse effect of fixation (Novikoff et al., 1953, 1958; and Essner et al., 1958). However, with liver that had been quenched with liquid nitrogen, sectioned and fixed in formalin-sucrose, Ashworth et al. (1963) were able to demonstrate ATPase activity in mitochondria as well as on the plasma membrane. Grossman and Heitkamp (1968) also pointed out that fixation inhibited ATPase activity, and were able to demonstrate Mg\(^{++}\)-ATPase activity in unfixed suspensions of mitochondria isolated from thigh skeletal muscle of rabbits.
In conclusion, then, 5'-nucleotidase was taken as a marker for plasma membrane fragments and G-6-Pase as a marker for ER fragments in biochemical studies concerning the separation of these fragments by zonal centrifugation of 'microsomal material'. Consideration will later be given to cytochemical observations on tissue fractions which correlate quite well with biochemical data on such fractions.

2. Zonal centrifugation of hepatic 'microsomal material'.

(a) The search for optimal conditions for separating membranous elements.

Zonal centrifugation of 'microsomal material', in the presence of Mg++, gave three main peaks of enzyme activity. The first region, covering fractions isolated at a density of less than 1.10 contained 'soluble' enzyme activities. Siekevitz (1962) pointed out that the term 'soluble' is a vague one and that mere resistance to centrifugation in a specified gravitational field is an arbitrary criterion for enzyme solubility. However, in the present context the 'soluble' region refers to those zonal fractions containing slowly sedimenting material. A peak for RNA was found in this region. Its position corresponded to an S value of less than 10, indicative of transfer RNA (soluble-RNA).

The second region possessed a peak of 5'-nucleotidase activity and a small peak of G-6-Pase activity, the latter peak being considerably enlarged in runs carried out in the absence of Mg++. The effect of Mg++ on the centrifugal behaviour of membrane fragments is considered later. The density of material recovered from this second or 'central'
region ranged from 1.10 to 1.17. Since 5'-nucleotidase and G-6-Pase exhibited overlapping densities in this region it was considered advantageous to divide it by two cuts, into further regions. The first, of density between 1.10 and 1.16, possessed a high 5'-nucleotidase activity with negligible G-6-Pase activity and consisted mainly of smooth surfaced membrane fragments. It was termed the 'smooth' region. The second cut of the 'central' region, incorporating material isolated at a density ranging from 1.16 to 1.18, also exhibited considerable 5'-nucleotidase activity but its G-6-Pase content was higher. This intermediate region contained smooth surfaced vesicles but also some rough surfaced vesicles.

The third region, generally that region of the gradient having a density greater than 1.17, contained most of the G-6-Pase activity and RNA of the material loaded into the zonal rotor. It also showed a peak of 5'-nucleotidase activity. This region was termed the 'rough' region because it contained rough surfaced membrane fragments.

At the commencement of this study, density values ranging from 1.05 to 1.18 for smooth vesicles and a value of 1.20 for rough vesicles had been reported (Dallner, 1963; Emmelot et al., 1964b; El-Aaser et al., 1966b; Rothschild, 1963; Song et al., 1967). As early as 1956 Palade and Siekevitz pointed out that lipid-rich membrane fragments were lighter than ribosomes which were low in lipid.
Ribosomes have a density of 1.5 (Peterman, 1964). The density values mentioned above indicated that plasma membrane fragments and smooth ER fragments have overlapping densities. Confirmation of this latter point was obtained when enzyme activities of zonal runs were recalculated to be linear with the density of the gradient. 5'-Nucleotidase then exhibited activity free from G-6-Pase activity at a density ranging from 1.14 to 1.15, while the two enzymes showed overlapping activities at a density of 1.17.

Bearing in mind the overlapping densities of plasma membrane and smooth ER fragments an attempt was made to separate such fragments by altering the shape of the gradient. Sigmoid gradients, usually plateauing at a density of 1.17, were tried and they gave better separations than had been previously achieved with linear gradients. Thus in run 29 which had a linear type gradient the AMPase was spread over the 'central' region. In run 30 with a sigmoid gradient the 'central' peak of AMPase activity was sharper, but because of the steepness of the gradient (see run 26 also) the 'soluble' and 'central' regions ran very close together. In both runs AMPase exhibited peaks at a density ranging from 1.12 to 1.15. In run 30 Mg++-activated ATPase showed a broader peak than that observed for AMPase and, moreover, was equally divided between the 'smooth' and 'intermediate' regions, whereas greatest enrichment in AMPase activity was in the 'smooth' region. This relatively high ATPase activity in the 'intermediate' region may be attributable to mitochondrial fragments contaminating the region. Mention has
already been made of the common failure to demonstrate mitochondrial ATPase activity. Nevertheless, Mg\textsuperscript{++}-
activated ATPase activity has been cytochemically demonstrated in the plasma membrane and in mitochondria
(Ashworth et al., 1963; Grossman and Heitkamp, 1968).

In run 30 peaks for RNA closely paralleled those for G-6-Pase. A peak was observed at a density of 1.07,
this being attributable to soluble RNA, while membrane-bound ribosomes [Fig. 8(d)] account for the RNA peak at
density 1.2. Since ribosomes have a density of 1.5, the small peak of RNA noted at a density of 1.15 probably
represents free ribosomes sedimenting through the gradient. However, membrane-bound ribosomes have sometimes been
observed in electron micrographs of similar fractions from other runs [see Fig. 8(a)].

Runs 26 and 27 further illustrate the point that the position of the enzyme peaks depends on the shape of the
gradient. The steeper gradient in run 26 gave sharper peaks of activity for AMPase and for ATPase at a density of 1.15
than that observed for run 27 where AMPase was spread over the 'central' region.

Different homogenization media were tried in the hope of attaining improved separation of plasma membrane fragments
from ER fragments. With initial use of 0.12 M sucrose, 0.25 M sucrose, or 0.14 M NaCl, quite similar proportions of
5'-nucleotidase activity were recovered in the zonal 'central' region from 'microsomal material' in the presence
of Mg$^{++}$ [Table 1; runs 30, 27 and 31]. Nevertheless, with 0.12 M sucrose there was some increase in 5\'-nucleotidase activity in the 'smooth' region [Table 2b; runs 29 and 30]. No advantage was gained by employing 0.14 M NaCl as the homogenization medium. It was hoped that saline would lessen the risk of membranes adhering to one another, as may happen with sucrose solutions. However, the initial use of saline resulted in an increase in protein and enzyme activities recovered in the 'soluble' region.

Increasing the time of zonal centrifugation from 3 to 9 hours had no noticeable effect on the proportion of AMPase recovered in the 'central' region. There was a slightly higher recovery of enzyme activity in the region of higher density ($D_{20}>1.17$). Nevertheless, it seems likely that an isopycnic pattern for AMPase was achieved after 3 hours of zonal centrifugation.

An attempt was made to achieve further purification by recentrifuging zonal fractions on continuous sucrose gradients. Most regions, from zonal runs carried out in the presence of Mg$^{++}$, gave a single band but the 'central' region separated into two bands. The upper band, isolated at a density of 1.18, contained all the enzyme activities of the 'central' region and consisted of some small sheets of membrane and many vesicles. This band possessing 5\'-nucleotidase and G-6-Pase activities presumably consists of plasma membrane and ER fragments. The lower band, isolated at a density of
1.29, lacked in enzyme activity but consisted of vesicles rather similar in appearance to those found in the upper band. Fragments of the Golgi apparatus or other cellular organelles might account for such vesicles. However, it is possible that the vesicles in the lower band are in fact of plasma membrane or ER origin and that their lack of enzyme activity is due to inhibition by the high concentration of sucrose, which is known to inhibit phosphatases (Hinton et al., 1969), in the band.

By rupturing Ehrlich ascites cells in a 'nitrogen pressure homogenizer' (Hunter and Commerford, 1961) and by density-gradient centrifugation in either sucrose or Ficoll, Wallach and Kamat (1964) separated plasma membrane vesicles from those derived from the ER. These authors found that the two classes of vesicles responded differently to changes in ionic and osmotic environment. The addition of Mg$^{++}$ produced an increment in the density of ER vesicles while affecting plasma membrane vesicles to a much smaller degree.

Zonal centrifugation of 'microsomal material', prepared by initial homogenization of a parenchymal cell suspension in a 'nitrogen pressure homogenizer' showed a good separation of G-6-Pase-containing elements from AMPase elements. A sharp peak of AMPase activity was found at a density of 1.13. It seems likely that in this run the plasma membrane fragments had not reached their isopycnic position in the gradient after two hours of zonal
centrifugation. The bulk of the G-6-Pase activity was recovered in the higher density region (Table 1). That the 'nitrogen pressure homogenizer' causes severe fragmentation of the plasma membrane (Wallach and Kamat, 1964) with an increase in the recovery of these fragments in the microsomal fraction was confirmed by Hinton et al. (1967) who found that use of such a homogenizer resulted in a fall in the normally large amount of 5'-nucleotidase recovered in the crude nuclear fraction. The results of this experiment indicate the usefulness of the 'nitrogen pressure homogenizer' in work concerning the separation of plasma membrane and ER fragments. An electron microscopic and cytochemical examination of zonal fractions from runs similar to that illustrated in Fig. 16 might be beneficial, since fragments of the plasma membrane which normally would sediment with the nuclear fraction, viz., fragments of the bile canaliculus, may well have been so damaged as to sediment with the microsomal fraction.

No separation of 5'-nucleotidase-containing fragments from those possessing G-6-Pase activity was achieved with zonal runs carried out in the absence of Mg\(^{++}\) (see Table 1). In a zonal run in which the gradient was buffered with bicarbonate, no difference was observed in the distribution of enzymes when compared to runs in which the gradient was buffered with Tris.
Zonal runs were tried with Mg$^{++}$ added to the homogenization medium and to the solutions of the gradient. It was hoped that divalent cations would selectively alter the centrifugal behaviour of certain types of membrane fragments (Dallner, 1963; Wallach, Kamat and Gail, 1966). In all zonal runs separation of AMPase from G-6-Pase was achieved only in the presence of Mg$^{++}$ (see Table 1). In most runs the concentration of Mg$^{++}$ was 0.005 M. Raising the concentration to 0.01 M caused no marked change in the position of the enzyme peaks. However with the Mg$^{++}$ concentration at 0.01 M there was a decrease in the recovery of AMPase activity in the 'central' region and an increased recovery in the higher density region (Table 1; runs 33 and 34), indicating that the higher concentration of Mg$^{++}$ caused an increase in the density of the plasma membrane fragments. This is in disagreement with the findings of Wallach et al. (1966) who found that with Ehrlich ascites cells the addition of Mg$^{++}$ produced a large increment in the density of ER vesicles while affecting plasma membrane vesicles to a much smaller degree, but it must be borne in mind that these authors used Mg$^{++}$ at a concentration of 0.001 M.

No separation of AMPase from G-6-Pase was achieved with a zonal run carried out in the presence of Cs$^+$ in place of Mg$^{++}$. Dallner (1963) stated that the density gradient centrifugation, in the presence of Cs$^+$ (15mM), of a '10,000 g supernatant' from rat liver gave rise to a double-layered fraction and a pellet consisting of rough surfaced vesicles and free ribosomes. Dallner claimed that Cs$^+$ binds preferentially to ribosomes. In run 19 (Fig. 14) peaks
for RNA in fractions 17 and 24 are probably attributable to free ribosomes sedimenting through the gradient. It seems likely that the concentration of Cs$^+$ (0.005 M) was not sufficiently high to increase the density of ribosomes, whether free or membrane-bound.

Zonal centrifugation of 'microsomal material' in the presence of Mg$^{++}$ (0.005 M) and Cs$^+$ (0.01 M) did not give any improved separation of AMPase and G-6-Pase when compared to runs carried out in the presence of Mg$^{++}$ alone.

An attempt was made to separate smooth surfaced membranes from rough surfaced membranes by using sodium deoxycholate, which is known to detach ribosomes when used in low concentration (Ernster et al., 1962). Deoxycholate at a final concentration of 0.5% caused a marked increase in the amount of 5'-nucleotidase recovered, in the 'soluble' region. This solubilizing effect of DOC, at a concentration of 0.5%, on 5'-nucleotidase is in close agreement with the results of Song and Bodansky (1967) who found that DOC at a concentration of 0.5% caused a release of 85% of total AMPase activity into the supernatant fraction of a human liver homogenate.

More than 50% of the G-6-Pase activity was recovered at a density greater than 1.17 (Table 1; run 18). In the same run 40% of the ATPase activity was recovered in the 'central' region as against 24% for 5'-nucleotidase. These results indicate that G-6-Pase and ATPase are more firmly bound enzymes than 5'-nucleotidase, in agreement with the observations of Dallner (1963). El-Aaser (1965) has also
shown that with microsomal fractions, 5'-nucleotidase is more readily solubilized by DOC than is G-6-Pase. Decreasing the concentration of DOC to 0.25% (run 20) caused a decrease in the amount of 5'-nucleotidase recovered in the 'soluble' region (Table 1, compare run 20 with run 18). In the present study no separation of plasma membrane fragments from ER fragments was achieved by using DOC, at least at the concentrations tried. Lower concentrations of DOC may prove more advantageous for such separations.

(b) Distribution of nucleoside di- and triphosphatases amongst zonal fractions.

There was an increase in the activity of ADPase in the 'soluble' region when compared with that found for AMPase and ATPase in the same region (Tables 1, 2a and 2b). The high recovery of ATPase in the 'soluble' region of run 31 is probably due to the solubilizing effect of saline, the use of which resulted in an increase in the 'soluble' activity of all the phosphatases studied. Dallner (1963) pointed out that nucleoside triphosphatase was not simply enclosed in vesicles but was bound to membranes by chemical forces. The distribution of 'non-soluble' ADPase activity follows closely that found both for 5'-nucleotidase, in agreement with the observations of Wattiaux and Wattiaux-De Coniock (1969), and for Mg$^{++}$-ATPase. This indicates that ADPase is a plasma membrane enzyme. ADPase and Mg$^{++}$-ATPase have been cytochemically demonstrated in the plasma membrane of rat liver (Goldfischer et al., 1964). The high recovery
found for ATPase loaded into the rotor may be due to an activation of the enzyme during the zonal centrifugation procedure.

UTPase activity was almost evenly distributed amongst the three zonal regions in run 31. The homogenization medium in this run was 0.14 M NaCl. As can be seen from Table 1, saline causes a greater release of UTPase into the 'soluble' region than that found for ATPase. Of relevance to this latter point is the suggestion by El-Aaser et al. (1966b) that UTPase and ATPase may not be merely common activities of the same enzyme, despite evidence for their identity (Goldfischer et al., 1964).

The distribution pattern for UDPase, as outlined in Tables 2a and 2b, showed that the greatest recovery of this enzyme activity was in the 'soluble' region. However, the distribution of the 'non-soluble' activity exhibited a pattern intermediate between that of AMPase and G-6-Pase. This is in agreement with the cytochemical observations of Goldfischer et al. (1964) and El-Aaser (1965) who demonstrated UDPase activity in the plasma membrane and in ER membranes.

The distribution of the 'non-soluble' IDPase activity was similar to that found for UDPase (see Table 2b and Figs. 7, 11 and 16). In agreement with this distribution pattern Goldfischer et al. (1964) cytochemically demonstrated IDPase activity in the plasma membrane and ER membranes. From the distribution of IDPase activity amongst subcellular fractions
from rat liver, Novikoff and Heus (1963) concluded that the enzyme was localized in the ER. Since they had previously shown (Novikoff et al., 1962) that activity occurred in the bile canaliculi it is surprising that the plasma membrane was not considered as a location site. In recent work with rat liver fractions, Wattiaux and Wattiaux-De Coniock (1969) found that IDPase was recovered mainly in the microsomal fraction and that its distribution was similar to that of G-6-Pase. They stated that purified plasma membrane preparations were greatly enriched in ADPase and AMPase but exhibited low IDPase activity and they concluded that IDPase was located in the ER.

In the work reported here, considerable IDPase activity was recovered in association with G-6-Pase at a density of about 1.20. Nevertheless, IDPase activity was also recovered in the 'central' region in association with 5'-nucleotidase. Sabatini et al. (1963) and Wood (1967) cytochemically demonstrated IDPase activity in the membranes of the Golgi apparatus. As it seems quite possible the Golgi vesicles sediment with the microsomal fraction of liver homogenates, some of the IDPase activity noted in 'microsomal material' might be due to the presence of such vesicles.
Novikoff et al. (1958) examined a tissue fraction for enzymic activity (ATPase, in a crude nuclear fraction) with the light microscope. However, it is only in recent years that the electron microscope has been applied to the enzymic study of tissue fractions, this probably being due to the difficulty involved in handling such fractions during and after incubation. As will be mentioned in the next Section, fixation of tissue fractions with glutaraldehyde results in a marked inhibition (biochemically and cytochemically) of phosphatases; this being in contrast to the acceptable cytochemical results obtained with sections cut from fixed liver blocks. Incubation of unfixed fractions in cytochemical media would be expected to give poor morphological preservation of membrane fragments and might give rise to diffusion of lead phosphate deposits.

One of the drawbacks of incubating tissue fraction suspensions, at least in conventional cytochemical assay media, is the risk of aggregation of the membrane fragments by the high concentration of lead, a factor which would give rise to poor morphological preservation of such fragments. Another snag envisaged at least with work on microsomal suspensions would be the length of time required to 'pellet' such suspensions, after completion of incubation, for electron microscopic processing. Unless the enzyme reaction is completely stopped the suspension would be expected to overstain during the centrifugation procedure.
The cytochemical examination of fractions by El-Aaser (1965) entailed the incubation of pellets. Whereas the techniques for morphological examination of pellets are straightforward, certain problems arise when such pellets are cytochemically examined to reveal enzyme activities. As with tissue blocks the main obstacle is the poor and variable penetration of pellets by substrates which may give rise to artefactual staining patterns.

Evidently it was desirable to develop techniques for the preparation of thin sections from tissue fractions. Acceptable cytochemical results were obtained when incubation was performed with thin sections prepared by a novel technique, and also with thin pellets. The merits of such techniques are discussed later. The work now to be considered had to go hand in hand with zonal work (see Section B.2 below) which would have benefited from good cytochemical techniques at the outset.

(a) **Effects of glutaraldehyde and lead ions on enzyme activities.**

It was hardly to be expected that cytochemical work could be done on unfixed tissue. In reviewing the preservation of cellular ultrastructure together with biochemically assayed enzymic activity by aldehyde fixation, Sabatini *et al.* (1963) concluded that glutaraldehyde gave the best general preservation of cellular fine structure. In the present study of phosphatases in liver sections, glutaraldehyde was the chosen fixative. Although glutaraldehyde is known to cause marked inhibition of phosphatase enzyme activities, as is
formaldehyde (Tice and Engel, 1966; Marchesi and Palade, 1967; Reale and Luciano, 1967) the residual activity was sufficient to allow demonstration of enzyme activities, at least in sections cut from whole tissue as opposed to tissue fractions (see below).

Anderson (1967) studied the effects of glutaraldehyde on enzyme activities and concluded that the inhibition of such enzymes was due to the presence of impurities in commercial glutaraldehyde. In agreement with the observations of Anderson (1967) the spectral absorption curve for purified glutaraldehyde, purified by repeated washing with charcoal, showed an absorption maximum at 280 mÅ. On the other hand, commercial glutaraldehyde exhibited a small peak at 280 mÅ with a second absorption maximum at 235 mÅ, this being attributable to impurities. The effects of glutaraldehyde, including a purified preparation, on 5'-nucleotidase and G-6-Pase activities were as shown in Table 4. Glutaraldehyde caused marked inhibition of enzyme activity, with little difference in the inhibiting effects between commercial and purified glutaraldehyde. G-6-Pase, which is reported to be rapidly inactivated by glutaraldehyde (Orrenius and Ericsson, 1966), was inhibited to a lesser extent than 5'-nucleotidase. In line with these biochemical observations no activity could be cytochemically demonstrated for 5'-nucleotidase, G-6-Pase or ATPase in a microsomal pellet prefixed in 3% commercial glutaraldehyde. When the concentration of commercial glutaraldehyde was lowered to 1.5%, 5'-nucleotidase activity was demonstrable in a fixed microsomal pellet. However, staining may have reflected the longer incubation time used
in this experiment. A small proportion of vesicles gave a positive reaction for G-6-Pase and for ATPase in a microsomal pellet fixed in 1.5% commercial glutaraldehyde.

Whereas commercial and purified glutaraldehyde were quite similar in inhibiting 5'-nucleotidase and G-6-Pase in biochemical assays, purified glutaraldehyde gave more acceptable cytochemical results with pellets. With a plasma membrane preparation (possessing some G-6-Pase activity) which had been fixed in 3% purified glutaraldehyde, 5'-nucleotidase activity was demonstrable. No activity was noted with commercial glutaraldehyde even though the fixation time was half that used in the experiment with purified glutaraldehyde.

G-6-Pase activity was demonstrated in a semi-purified plasma membrane preparation (similar to that mentioned above) fixed in 3% purified glutaraldehyde. It was noted that the proportion of membrane fragments exhibiting G-6-Pase activity was greater than the proportion exhibiting 5'-nucleotidase activity. Possible explanations are that the incubation time was longer for G-6-Pase, that purified glutaraldehyde is less inhibitory towards G-6-Pase than towards 5'-nucleotidase (Table 4); alternatively, the preparation may have been richer in ER fragments than in plasma membrane fragments despite evidence to the contrary from biochemical assays.

From the biochemical and cytochemical observations on pellet fractions fixed in glutaraldehyde it was concluded that fixation was of no advantage. The specificity of the
staining reactions for 5'-nucleotidase and G-6-Pase (see below) and the reasonable morphological preservation of membrane fragments in unfixed preparations indicated that acceptable cytochemical results could be obtained with the omission of fixation prior to incubation. However, fixation times of one and two hours as used in this exploratory work on tissue fractions may have been excessive. Fixation in glutaraldehyde for shorter periods of time (up to 5 minutes) might be better, and is now being tried in this laboratory.

Lead ions, which act as trapping agents for released phosphate in cytochemical assays for phosphatases, are known to inhibit phosphatase activities (Novikoff et al., 1958; El-Aaser, 1965; Engel and Tice, 1966). The concentration of lead conventionally used in cytochemical assays resulted in about a 50% decrease in 5'-nucleotidase, G-6-Pase and ATPase activities in biochemical assays (Table 5). Evidently the use of lead ions at this concentration, coupled with the use of glutaraldehyde fixation, militates against the demonstration of little cytochemical activity with fixed tissue fractions. Lowering the concentrations of lead ions reduced the inhibition of enzyme activities (Table 5) and even, in some instances, increased the activity. However, such low concentrations of lead are known to be insufficient to trap all the released phosphate, and may cause artefactual staining (light microscopy studies) due to diffusion of lead phosphate, at least in work on liver sections (Novikoff et al., 1958; Reid et al., 1964; El-Aaser, 1965). With tissue fractions the use of lead at low concentrations might be permissible and
advantageous in cytochemical studies. This matter will 
be taken up in future studies.

The techniques of enzyme cytochemistry, at the level 
of the electron microscope, are still subject to rigorous 
criticism on the grounds of false localizations. Since 
commencement of this work it has been reported that lead 
ions in the Wachstein-Meisel medium cause the non-enzymic 
hydrolysis of ATP and other nucleoside phosphates 
(Rosenthal et al., 1966; Moses et al., 1966; Moses and 
Rosenthal, 1967, 1968). In this present study it was found 
that lead ions, at the different concentrations outlined 
in Table 5, did not cause any non-enzymic hydrolysis of 
UMP, ATP or G-6-P. This is in agreement with the observations 
of Novikoff (1967) who repeated and extended the experiments 
of Rosenthal et al. (see above) and concluded that the 
staining reactions obtained in the Wachstein-Meisel medium 
and similar media reflected real enzymic activities rather 
than non-enzymic hydrolysis.

In work on the localization of ATPase activity in 
striated muscle, Gillis and Page (1967) studied the 
distribution of lead phosphate grains precipitated by adding 
phosphate solutions to fibrils which had been incubated with 
lead in the absence of ATP. Whilst the grains were not 
uniformly distributed along the fibrils, they showed many 
of the features of the distribution pattern found on 
incubating the fibrils with ATP and lead. The authors 
suggested that differential lead binding by the protein 
filaments or movement of the grains, may have been responsible
for the non-uniform distribution, and that consequently no conclusions concerning sites of ATPase activity within the sarcomere could be drawn from work entailing lead precipitation.

The non-specific deposition of lead phosphate observed in cytochemical studies on blood platelets by Behnke (1966) and on human liver by Wills (1967) led these authors to conclude that lead-containing media were not suitable for visualizing fine structural localization of enzymic activities.

Cytochemical experiments reported in this thesis were accompanied by controls in which the substrates were replaced with water. In none of these experiments, involving both liver sections and fractions, was deposition of lead detected [Figs. 25(d) and 28(e)]. This indicates that the staining reaction is valid, at least with liver, since deposition of lead phosphate in the absence of substrate has been reported for other tissues (see above).

(b) Cytochemical studies on a crude microsomal fraction.

The variety of membranous elements observed in a microsomal fraction by Palade and Siekevitz (1956) led them to conclude that the fraction was not homogenous from a cytological standpoint. Their electron microscopic examination of the microsomal fraction revealed the presence of rough surfaced vesicles bearing morphological resemblances to the rough surfaced elements of the ER of hepatic cells.
In addition to the rough surfaced membranes the fraction contained a small number of vesicles of a smooth surfaced type probably derived from the smooth surfaced region of ER or the plasma membrane (Palade and Siekevitz, 1956). Since plasma membrane fragments sediment mainly in the nuclear and microsomal fractions of liver homogenates, as judged by the recoveries of 5'-nucleotidase (a plasma membrane marker) in both fractions (de Lamirande et al., 1958; Segal and Brenner, 1960; Novikoff and Heus, 1963; Reid et al., 1964; El-Aaser and Reid, 1965), a cytochemical examination of a crude microsomal fraction was carried out in order to try out procedures that might help in the identification of plasma membrane fragments and to distinguish them from ER fragments. The results obtained from the cytochemical examination of membrane fractions obtained by zonal centrifugation of a crude nuclear fraction and a microsomal fraction are discussed later.

As already pointed out, it is difficult to handle tissue fractions for enzyme cytochemical studies at the electron microscope level. Acceptable cytochemical results, as now reported, were obtained when thin 'sections' prepared by method 'A' were incubated in media to demonstrate phosphatase activities. This method consisted in layering a microsomal suspension on glass slides (in the cold) to give a thin film which was peeled off, resembling a thin section. The results of incubating thin 'sections' of microsomal material ('sections' prepared using method 'A') in media to demonstrate phosphatase activities were as shown in Fig. 25. 5'-Nucleotidase activity was demonstrated on sheets of membrane and on
some vesicles while only a few vesicles exhibited ATPase activity. G-6-Pase activity was demonstrated on membranous elements differing in morphological appearance from those exhibiting 5'-nucleotidase activity. The fact that only a small proportion of membrane fragments exhibited G-6-Pase activity may be due to inhibition of enzyme activity by the high concentration of sucrose (1 M) used in preparing the thin sections (sucrose was not added to the incubation medium), or to a lag period in lead phosphatase deposition due to G-6-Pase. Further comment on this possible lag period will be made later in the Discussion.

The membrane fragments exhibiting 5'-nucleotidase activity are presumably derived from the plasma membrane. Homogenization of liver results in the fragmentation of the plasma membrane. As will be discussed later, large sheets of membrane and structures bearing resemblances to bile canaliculi sediment with the crude nuclear fraction. Small sheets of membrane and vesicles derived from the microvilli of the sinusoidal wall and the bile canaliculus would sediment with the microsomal fraction and account for the 5'-nucleotidase activity found in such fractions. On a morphological basis no distinction could be made between vesicles derived from the plasma membrane (5'-nucleotidase-containing vesicles) and those derived from the smooth ER (G-6-Pase-containing vesicles), at least in the work reported here. However, since ER membranes are reckoned to be thinner than the plasma membrane (Palade and Siekevitz, 1956; Tashiro, 1957) high resolution electron microscopy should help in
distinguishing the two types of membrane, as should the
fact that after bleaching of liver sections, phosphotungstic
acid at low pH specifically stains the plasma membrane of
liver cells (Emmelot et al., 1964a).

To obtain evidence on the specificity of staining in a population of microsomal vesicles, thin sections were incubated for varying periods of time in media to demonstrate 5'-nucleotidase and G-6-Pase activities. A freezing microtome was employed to cut 30µ sections from a frozen unfixed microsomal pellet. The results of incubating sections prepared by this method, method 'B', compared favourably with those obtained by incubating 'sections' prepared by method 'A'.

Incubation studies revealed no increase with time, from minutes to hours, in the proportion of membrane fragments exhibiting 5'-nucleotidase activity. However, an increase in the intensity of the staining reaction after 2 hours of incubation accompanied by a deterioration in the morphology of the membrane fragments was noted.

The results of incubating sections for G-6-Pase showed that the intensity of the staining reaction increased dramatically when the incubation time was increased. This is in contrast to the results obtained for 5'-nucleotidase, at least for incubation times up to 30 minutes. The greater intensity of the G-6-Pase staining after 15 minutes with that after 5 minutes is indicative of a lag phenomenon with G-6-Pase. This lag period might account for the paucity
of vesicles exhibiting G-6-Pase activity when a section from a microsomal pellet, biochemically rich in G-6-Pase, was incubated for 5 minutes [Fig. 25(c)]. With sections incubated for G-6-Pase for 30 minutes or 1 hour, the majority of membrane fragments gave a positive reaction, as was the case after 15 minutes incubation, but a deterioration in their morphology was noted.

2. Cytochemistry of zonal fractions.

(a) Cytochemical observations on fractions isolated by zonal centrifugation of a crude nuclear fraction.

A plasma membrane fraction which was isolated by zonal centrifugation of a crude nuclear fraction from liver (El-Aaser et al., 1966a) revealed the presence of large irregular sheets of membrane and some vesicles when examined under the electron microscope. Since the fraction was rich in 5'-nucleotidase activity in biochemical assays, a cytochemical examination of the fraction was carried out to assist in the identification of the membrane fragments possessing such activity and to check the cytochemical procedures.

One such fraction, enriched in 5'-nucleotidase activity but possessing considerable G-6-Pase activity and thus not a pure plasma membrane fraction, was cytochemically examined to demonstrate both enzyme activities. In this experiment thin sections were prepared by using method 'A' and acceptable cytochemical results were obtained.
5' - Nucleotidase activity was demonstrated on large sheets of membrane presumably derived from the plasma membrane but not on vesicular membrane fragments resembling those derived from the ER or swollen mitochondria. G-6-Pase activity in the fraction gave a pattern similar to that obtained for a liver section (see Fig. 3), the lead phosphate deposits being localized to the nuclear envelope and to membrane fragments of the ER.

When a relatively pure zonal plasma membrane fraction (unlike the fraction considered above) was cytochemically examined for 5'-nucleotidase and G-6-Pase the results were as shown in Figs. 31 and 32. In this experiment thin pellets were incubated (method 'C'). Although acceptable cytochemical results were obtained, it cannot be ruled out that there may have been artefactual staining caused by the different rates of penetration of the different substrates into such pellets. When a plasma membrane pellet was incubated for 5'-nucleotidase, many sheets of membrane and structures resembling bile canaliculi gave a positive reaction quite specifically. The lack of activity in mitochondria and lysosomes shows that it is not a matter of non-specific absorption of lead phosphate to membrane fragments. It is of interest to note that with unfixed tissue fractions lysosomes did not stain for 5'-nucleotidase, as was the case with fixed liver sections. This lack of activity in lysosomes and also in many sheets of membrane might be due to some hindrance in access by the substrate.
Alternatively, activation of lysosomal non-specific acid phosphatase activity, attributable to an alteration in the permeability of the lysosomal membrane after fixation, might account for the activity found in liver sections.

Emmelot et al. (1964a) isolated from rat liver a plasma membrane fraction which consisted of sheets of membrane similar in appearance to those shown in Fig. 32. These authors stated that G-6-Pase activity (assayed biochemically) was present in the plasma membrane. In this study a zonal plasma membrane fraction incubated to cytochemically demonstrate G-6-Pase activity showed no activity on sheets of membrane and on structures resembling bile canaliculi. However, small vesicles adhering to the sheets of membrane (presumably plasma membrane) gave a positive reaction. It seems likely that these vesicles, which are probably derived from the ER or nuclear envelope and which adhere to the plasma membrane, account for the G-6-Pase activity as found in plasma membrane preparations by Emmelot et al. (1964a). Takeuchi and Terayama (1965) isolated a plasma membrane fraction from a crude nuclear fraction by differential centrifugation on sucrose gradients. An electron microscopic examination of their plasma membrane fraction revealed the presence of sheets of membrane. They detected small vesicles adhering to the sheets and stated that such vesicles probably originated from microbodies or from the ER.
In an attempt to achieve further purification, a zonal plasma membrane fraction was recentrifuged on a discontinuous sucrose gradient. This method yielded a purified fraction rich in 5'-nucleotidase activity and low in succinic dehydrogenase (a mitochondrial marker), and also a second fraction which possessed both activities. When pellets were incubated, 5'-nucleotidase activity was localized to the majority of the membrane fragments, both sheets and vesicles, but there was a difference in the intensity of the staining reaction within the section finally prepared. As mentioned previously, this might be due to a variable penetration of such pellets by the substrate. When the second fraction (see above) was incubated for 5'-nucleotidase, deposition of lead phosphate occurred on sheets of membrane [Fig. 33(b)] similar in appearance to those exhibiting activity in the purified fraction.

(b) Cytochemistry of fractions obtained by zonal centrifugation of microsomal material.

5'-Nucleotidase and G-6-Pase were cytochemically demonstrated in fractions from the 'central' region of a run similar to that of run 32 or 27 but done with a 'B-XV' zonal rotor. 5'-Nucleotidase activity in the 'smooth' and 'intermediate' regions was demonstrated on small sheets of membrane and on vesicles presumably derived from plasma membrane microvilli. Small fragments of membrane exhibiting 5'-nucleotidase activity and bearing resemblance to desmosomes were sometimes observed. G-6-Pase activity was cytochemically demonstrated in the 'intermediate' region, with deposition of lead phosphate on vesicles. However,
small sheets of membrane which gave a positive reaction for 5'-nucleotidase did not do so for G-6-Pase.

As can be seen from Figs. 35 to 37, many vesicles gave a negative reaction for both 5'-nucleotidase and G-6-Pase. Although this may be due to a defect in the cytochemical technique, it is conceivable that there are vesicles which are derived from membranes other than the plasma membrane or ER, and which sediment in this 'central' region. Golgi vesicles and vesicles arising from cell organelles such as mitochondria, microbodies and lysosomes might well be present as contaminants in the 'central' region. Since monoamine oxidase, a marker for the outer mitochondrial membrane (Schnaitman et al., 1964) was detected in the central region, and since the density of this membrane, at least in human liver is in the range 1.12 to 1.14 (Song et al., 1967), it seems likely that outer mitochondrial membrane fragments are present along with fragments of the plasma membrane and ER in the 'central' region.

(c) Features of zonal fractions.

Biochemical, morphological and cytochemical findings already discussed, centered on 5'-nucleotidase and G-6-Pase, now warrant juxtaposition. In the case of the nuclear fraction, most of the membrane fragments exhibiting 5'-nucleotidase activity in cytochemical tests on plasma membrane preparations are in the form of large sheets, often accompanied by structures resembling intact bile canaliculi [Figs. 31 and 33]. The specificity of the 5'-nucleotidase
staining reaction was confirmed by the lack of activity exhibited by cellular organelles such as mitochondria and lysosomes [see Section 2 (a) above] and by the staining noted throughout the field in purified plasma membrane preparations.

On the other hand, small sheets of membranes and vesicles gave a positive reactions for 5'-nucleotidase [Figs. 35 and 36] when microsomal material was cytochemically examined. However, in contrast to results obtained with plasma membrane preparations from the nuclear fraction, no overall staining was observed with microsomal plasma membrane fractions. This is indicative of such fractions being contaminated with vesicles which are not of plasma membrane origin. Contamination by lysosomes was ruled out by the lack of acid phosphatase activity detected in biochemical assays on microsomal zonal fractions. Work at present being carried out in this laboratory is concerned with the isolation of a purified microsomal plasma membrane fraction by employing improved zonal techniques. Such a purified fraction would be expected to stain throughout for 5'-nucleotidase. G-6-Pase activity in nuclear and microsomal zonal fractions was localized to small vesicles presumably derived from the ER or from the nuclear envelope.

In future work concerning the cytochemical examination of tissue fractions it is hoped to improve the techniques involved in handling such fractions. Incubation of suspensions, as an alternative to sections, might be beneficial in view of the above remarks on the use of
pellets. Although acceptable cytochemical results were obtained with unfixed tissue fractions and in view of the phosphatase inhibition caused by fixation for long periods of time (1 to 2 hours) with commercial and purified glutaraldehyde, it is still a reasonable hope that fixation with purified glutaraldehyde [see Section 1 (a) above] for short period of time would give improved morphological preservation of membrane fragments when incubated in cytochemical media.

C. Studies on hepatoma.

1. Ultrastructure.

Pitot (1962) noted a change in morphological and enzymic properties between a primary hepatoma induced by ethionine and successive transplants from the same tumour. In this present study, sections from an ethionine-induced transplanted hepatoma, at different transplant generations, were examined under the electron microscope in order to compare their morphological characteristics.

With a 9th generation transplant (Fig. 17), the most notable ultrastructural characteristic when compared with normal liver (Fig. 1) was the lack of ER membranes arranged in parallel arrays in the former. In the hepatoma the nuclei appeared to be enlarged and the mitochondria more rounded than those observed in sections from normal liver. Cell contact appeared to be quite normal at least at an early stage of transplantation (Fig. 17). Derangements of ER membranes similar to those noted in Figs 17 and 18 have been observed in many hepatomas by other authors (Howatson and Ham, 1955; Behrnard, 1958; Mercer, 1961; Pitot, 1962; Ma and Webber, 1966; Merkow et al., 1967).
No marked difference in ultrastructure was observed in an ethionine-induced hepatoma after 44 generations of transplantation (Fig. 18) when compared with a hepatoma after 9 transplant generations (Fig. 17). However, in agreement with the observations of Emmelot et al. (1964a) a deterioration in cell contact was observed in a hepatoma section as illustrated in Fig. 18.

2. Marker enzymes.

In connection with the use of 5′-nucleotidase and G-6-Pase as marker enzymes for the plasma membrane and ER respectively in zonal centrifugation of hepatoma 'microsomal material', a cytochemical examination of both enzymes in hepatoma sections was carried out to check whether their intracellular localization differed from that of normal liver. There was also need to check reports that much of the 5′-nucleotidase activity of hepatoma, assayed biochemically and histochemically, seems to reside in connective tissue (Wachstein and Meisel, 1959; El-Aaser, 1965).

5′-Nucleotidase activity was demonstrated in the plasma membrane of a hepatoma section. Although the plasma membrane exhibiting 5′-nucleotidase activity in Fig. 19 may be that of a star cell, electron micrographs of other sections have revealed 5′-nucleotidase activity in the plasma membrane similar to that illustrated in Fig. 18.

G-6-Pase activity in a hepatoma section was as shown in Fig. 20. Because the ER membranes did not exhibit the highly organised pattern of membranes stacked in parallel arrays, as found in normal liver (see Fig. 3), it was more difficult to
recognise the intracellular localization of G-6-Pase in the hepatoma. Nevertheless, a positive reaction for G-6-Pase on the nuclear envelope and on membranous elements, presumably derived from the ER was observed.

Mg$^{++}$-activated ATPase, like 5'-nucleotidase, was cytochemically demonstrated in the plasma membrane of a hepatoma section. There was no evidence that the intracellular localizations of 5'-nucleotidase, ATPase and G-6-Pase in sections from an ethionine-induced transplanted hepatoma differed from those found in normal liver, and it was concluded that these enzymes could validly be employed as 'markers' in biochemical studies on hepatoma.

G-6-Pase activity was cytochemically demonstrated on the nuclear envelope in a section from an unfixed 15,000 g pellet prepared from a hepatoma homogenate. Although 5'-nucleotidase activity of hepatoma has been reported (see above) to reside in connective tissue, the cytochemical examination (as reported here) of a microsomal pellet prepared from a hepatoma homogenate showed activity in some small sheets of membrane and in vesicles. However, the absence of connective fibres exhibiting 5'-nucleotidase activity could be accounted for by the removal of such fibres during the initial filtration of the hepatoma homogenate. The level of G-6-Pase activity, assayed biochemically, in the ethionine-induced hepatoma had dropped to less than one-fourth that found for normal liver. This decrease in G-6-Pase activity may account for the paucity of vesicles exhibiting activity in hepatoma fractions. Good morphological preservation of hepatoma fractions was not attained, this probably being due to inadequate fixation.
3. Zonal centrifugation of hepatoma 'microsomal material' to separate membranous elements.

The distribution of phosphatases (Table 3) and RNA amongst 'microsomal' zonal fractions from an ethionine-induced transplanted hepatoma showed a pattern quite similar to that found for normal liver. Abnormally high activity (cf. Tables 1 and 2a) in the 'soluble' region was found for all the phosphatases studied in the hepatoma run. This increased 'soluble' activity might be due to hepatoma membranes being more fragile than normal liver membranes and to a release of their enzyme content during fractionation procedures. The high recovery for ADPase and for IDPase loaded into the rotor might be due to an activation of these enzymes during the process of zonal centrifugation. In work on normal liver Dallner (1963) stated that the high recovery found for nucleoside diphosphatase (UDPase and IDPase) was due to marked activation during fractionation.

In the hepatoma run illustrated in Fig. 23 the level of G-6-Pase, assayed biochemically, was one-fourth of that found for normal liver. Nevertheless the distribution of the remaining activity amongst the zonal fractions was similar to that observed for normal liver (Table 3). The 'non-soluble' activity of IDPase gave a distribution pattern intermediate between that of 5'-nucleotidase and G-6-Pase, as was the case with normal liver.
ADPase, and alkaline β-glycerophosphatase activity (assayed at pH 9.6) in the absence of Mg\(^{++}\) which was undetectable in normal liver, closely paralleled 5'-nucleotidase in hepatoma material.

Although possible localization in connective tissue was not considered, the distribution pattern for alkaline β-glycerophosphatase is such that it is a good plasma membrane marker in the hepatoma. In line with this Emmelot et al. (1964a) cytochemically demonstrated alkaline β-glycerophosphatase activity in the plasma membrane of a rat hepatoma.

When the zonal fractions were pumped from the rotor with monitoring of absorption at 280 nm, it was noted that the absorption peak at a density of about 1.2 was abnormally low (not illustrated in Fig. 23). This is indicative of a reduction in the amount of rough surfaced ER membranes in the ethionine-induced hepatoma. Confirming this, zonal fractions isolated at a density ranging from 1.20 to 1.22 (Fig. 23), consisted mainly of smooth surfaced membranes, although some rough surfaced membranes were also present [Fig. 22(c)]. Mercer (1961) mentioned that in the more anaplastic tumours there was a paucity of rough surfaced membranes but that free ribosomes were plentiful.

An electron microscopic examination of fractions from the 'central' region in the hepatoma run shown in Fig. 23 revealed the presence of smooth surfaced membranes and particles resembling free ribosomes. Free ribosomes sedimenting through the gradient probably account for the peaks of RNA in the 'central' region (see bottom graph in Fig. 23). The smooth surfaced membranes from the 'central' region of the hepatoma run appear
swollen when compared with smooth surfaced membranes from normal liver 'microsomal material' (compare Fig. 22 with Fig. 8). This again may be indicative of hepatoma membranes being more fragile than those from normal liver. In line with this, Oberling and Bernhard (1961) stated that tissue sections of cancer cells had more fragile mitochondria than normal cells. Alternatively, because of the poor cell contact of hepatoma cells (Emmelot et al., 1964a) and because cell breakage during homogenization is generally more difficult to achieve with tumours than with tissue such as liver (Novikoff, 1960) it is quite possible that larger vesicles, not necessarily swollen, are in fact 'pinched off' during homogenization procedures.

In this study hepatoma membrane fragments exhibited poor morphological preservation. Inadequate fixation, possibly due to hepatoma membranes differing in chemical composition from those of normal liver and having different fixative-binding properties might account for this poor morphology. However, the possibility also remains that the membrane fragments exhibiting poor morphological preservation were derived from necrotic areas of the hepatoma and as such would not be expected to reveal good morphological characteristics,
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