RELATIONSHIP BETWEEN RAT SERUM PROTEINS
AND HEPATOCELLULAR SURFACE MEMBRANE

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by

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TO THE MEMORY OF MY FATHER
SUMMARY

Changes in the level of serum enzymes, notably 5'-nucleotidase and alkaline phosphatase, have frequently been used as a diagnostic aid for liver disease. Therefore the relationship between rat serum proteins and liver plasma membrane was investigated using immunofluorescence and affinity chromatography.

The extent of binding of the isolated plasma membrane to fluorescein-labelled anti-(rat serum) globulin fraction was examined, and a valid assay for proteins homologous to serum was developed. About 1% of the protein of the globulin fraction was capable of binding to the membrane. Washing the isolated membrane with 0.15 M-NaCl and 0.2 M-NaHCO₃, pH 9.0, resulted in the removal of the non-membraneous proteins as well as some of the genuine membrane proteins. The proportion of anti-(rat serum)-binding material washed off is about the same as the proportion of the total membrane proteins that is solubilised. However, when the binding of the isolated and salt-extracted membranes to anti-(liver plasma membrane) antiserum was examined, it was found that the washing procedure caused the exposure of a new binding site which was previously masked.

Affinity chromatography of rat serum on immobilised anti-(plasma membrane) conjugate resulted in the isolation of serum proteins, about 4% of total serum protein, common to liver plasma membrane. Enzyme activity measurements showed that none of the 5'-nucleotidase and
Alkaline/phenylphosphatase was associated with the common proteins. However, the additional 5'-nucleotidase which appears in the serum of jaundiced rats was found to originate from the hepatocyte plasma membrane (Issa et al., 1976).

The microsomal fraction of rat liver homogenate was fractionated using a linear sucrose density gradient. The plasma membrane fragments recovered in the microsomal fraction were found to be heterogeneous in density. It was demonstrated that 5'-nucleotidase, the most commonly used plasma membrane marker, is concentrated in the material banding at a density of 1.14 g/ml while most of other plasma membrane markers were found at a density of 1.16 g/ml. The possibility that the light microsomal subfraction is derived largely from the blood-sinusoidal surface of the hepatocyte was confirmed by an in situ labelling with SITS.

Examination of serum proteins after injection of L-[^14C]fucose showed that the proteins common to liver plasma membrane and rat serum are labelled more slowly than other secretory proteins. In addition, plasma membrane fragments from the sinusoidal facing area of the liver cell seem to be labelled more rapidly than other plasma membrane fragments. The results provide evidence that the material reaching the sinusoidal part of the plasma membrane is either released into the blood directly or moves to other parts of the membrane, finally to the bile canalicular region.
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## ABBREVIATIONS

Not included in the Biochem. J. abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Alk PDase</td>
<td>alkaline phosphodiesterase assayed with bis-p-nitrophenylphosphate as substrate.</td>
</tr>
<tr>
<td>AMPase</td>
<td>5'-nucleotidase assayed with adenosine 5'-phosphate (AMP) as substrate.</td>
</tr>
<tr>
<td>APNPPase</td>
<td>alkaline phosphatase assayed with p-nitrophenylphosphate as substrate.</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin.</td>
</tr>
<tr>
<td>DOC</td>
<td>sodium deoxycholate.</td>
</tr>
<tr>
<td>G6Pase</td>
<td>glucose 6-phosphatase assayed with glucose 6-phosphate as substrate.</td>
</tr>
<tr>
<td>INT</td>
<td>2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride.</td>
</tr>
<tr>
<td>LNase</td>
<td>L-leucyl-β-naphthylamidase assayed with L-leucyl-β-naphthylamide as substrate as a subgroup of <a href="https://en.wikipedia.org/wiki/L-Aminopeptidase">leucine aminopeptidase</a> (LAP).</td>
</tr>
<tr>
<td>PBS</td>
<td>physiological phosphate buffered saline (0.15 M-NaCl/0.01 M-phosphate, pH 7.5).</td>
</tr>
<tr>
<td>PCA</td>
<td>perchloric acid.</td>
</tr>
<tr>
<td>POPOP</td>
<td>1, 4-di-[2-(4-methyl-5-phenyloxazolyl)]-benzene.</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole.</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease assayed at pH 7.8 in the presence of 5 mM-MgCl$_2$.</td>
</tr>
<tr>
<td>SITS</td>
<td>4-acetamide-4'-iso-thiocyanato-stilbene-2, 2'-disulphonic acid disodium salt.</td>
</tr>
<tr>
<td>Succ.Dase</td>
<td>succinate dehydrogenase assayed with INT as substrate</td>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid.</td>
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CHAPTER ONE

INTRODUCTION
1 : 1) SERUM ENZYMES AS AN AID TO CLINICAL DIAGNOSIS

The majority of chemical changes in the body are mediated by enzymes, which are essential constituents of the cells and may either remain intracellular or pass out of the cell into the extracellular fluid, into the bloodstream, or in case of ducted glands, into the secretion. Normal blood contains a number of enzymes, and the variation in their levels was suggested to be of value for clinical diagnosis. Diagnosis from changes in serum enzyme levels is possible only in so far as any changes specifically and sensitively reflect damage in a particular organ. Moreover, the method used to estimate serum enzyme levels must itself be specific and sensitive. To the clinician the specificity of changes in serum enzyme activity as a function of generalized or specifically localized lesions is of great interest. Measurements of serum enzymes have been shown to be of value in the diagnosis of different diseases, for example: hepatic, myocardial, pancreatic, prostatic and muscular diseases, and various cancers (e.g. Baron, 1973; Moss and Butterworth, 1974). The most widely assayed serum enzymes in clinical laboratories are listed in Table 1-1 (from the brochure "Enzymes in Medical Practice, C.F. Boehringer & Soehne GmbH, Mannheim). Other enzymes are also assayed, such as 5'-nucleotidase, aminopeptidases, transpeptidases and in some cases ribonucleases.
Table 1-1: Serum enzymes commonly assayed in clinical laboratories.

<table>
<thead>
<tr>
<th>Enzyme</th>
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<tr>
<td>Glutamate-pyruvate transaminase*</td>
<td>GPT</td>
</tr>
<tr>
<td>Glutamate-oxaloacetate transaminase*</td>
<td>GOT</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>GLDH</td>
</tr>
<tr>
<td>Creatinine phosphokinase</td>
<td>CPK</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td></td>
</tr>
<tr>
<td>α-Amylase</td>
<td></td>
</tr>
<tr>
<td>Aldolase</td>
<td></td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>LDH</td>
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</table>

* synonym: aminotransferase

Enzymes in blood have been classified by Hess (1963) into two groups, designated as "plasma specific enzymes" and "non-plasma specific enzymes". The first group includes those enzymes which have a very definite and specific function in plasma. Plasma is their normal site of action and they are present in it at higher levels than in most tissue cells. Examples are blood coagulating enzymes and lipoprotein lipase. The second group, the non-plasma specific enzymes, have no known physiological function in plasma. They are present in plasma at concentrations much lower than in certain tissues. This group of enzymes includes those which are released from tissues during disease, and thus show increased levels
Among the enzymes released into the circulating fluid in the course of hepatic disease are aminotransferases, phosphatases, aminotranspeptidases and aminopeptidases. The aminotransferases are known to be enzymes of the hepatic cell cytosol and are presumably released into the circulation on breakage of the plasma membrane (Bengmark et al., 1967). Phosphatases and aminopeptidases, notably 5'-nucleotidase, alkaline phosphatase and L-leucyl-β-naphthyl-amidase, are, on the other hand, present abundantly in the plasma membrane of the liver cells. Consequently the mechanism of release of these enzymes into serum is probably more complicated than simply due to the breakage of the plasma membrane. The diagnostic advantages of these enzymes and clinical application of alkaline ribonuclease (RNase, a high proportion of which is found in liver plasma membrane) are discussed in the forthcoming section. Information available on γ-glutamyltranspeptidase will also be considered. In the liver this enzyme has been demonstrated in the canaliculi of the parenchyma and the cells lining the fine biliary ductules (Rosalki, 1975). During liver disease the behaviour of this enzyme is rather similar to that of alkaline phosphatase and 5'-nucleotidase and is therefore also of diagnostic relevance.

a) The origin of serum alkaline phosphatase in liver disease

To have a clear picture of the behaviour of serum alkaline phosphatase during liver disease, it is worth examining the origin of
this enzyme in normal serum. Because of the great problem of obtaining fresh tissues from normal humans, most of the work describing the tissue origin of this enzyme has been carried out on experimental animals. These studies indicated that serum alkaline phosphatase originates from several sources, notably skeleton, intestine and liver. The evidence presented later in this section will show that bone and intestine contribute the major part of serum alkaline phosphatase, at least in normal rats and dogs, while little is derived from the liver.

Studies on the effects of diet upon intestinal and serum alkaline phosphatase revealed that similar dietary regimens have similar effects on the two enzymes (Tuba and Madsen, 1952; Tuba and Shaw, 1950). Madsen and Tuba (1952) studied the effects of a number of inhibitors on alkaline phosphatase of kidney, liver, intestine, bone and serum in fed and fasted rats. They concluded that in normal fasted rats the major portion of serum alkaline phosphatase is derived from the intestine, and one or more of the other tissues may contribute a small portion to the total serum alkaline phosphatase. They suggested that the increased serum alkaline phosphatase in fed, as compared to fasted rats is mainly due to the entry of the intestinal phosphatase into the circulation. Saini and Posen (1969) supported the latter suggestion and found that serum alkaline phosphatase in fed rats reacts with anti-(rat intestinal-alkaline phosphatase). In the case of fasted rats, however, these authors demonstrated that serum alkaline phosphatase was virtually unaffected by anti-(rat intestinal-alkaline phosphatase), and hence
must originate elsewhere in the body. Recently Righetti and Kaplan (1971) confirmed this view, and reported that bone is the major source of alkaline phosphatase in serum of normal fasted rats. They compared the properties of the purified enzymes from liver, bone, intestine and serum from fed and fasted rats. These authors used the electrophoretic mobilities, effects of neuraminidase digestion, Michaelis constants, pH-optima and rates of urea and heat denaturation as criteria of the origin of the serum enzyme. They found that serum alkaline phosphatase in normal fasted rats originates from bone since, a) both bone and serum enzymes migrate identically on polyacrylamide gel electrophoresis, b) their electrophoretic migration rates are equally slowed after neuraminidase digestion, and c) both have similar Michaelis constants, pH optima and rates of urea and heat denaturation.

In liver disease, elevated levels of serum alkaline phosphatase have been associated with obstructive jaundice for more than 40 years (Roberts, 1930). In 1935, Armstrong and Banting suggested that the increased serum alkaline phosphatase in obstructive jaundice is of bone origin and that its accumulation in the blood is due to interference with its excretion in the bile. However, Freeman and Chen (1938) transfused normal dogs with blood of high alkaline phosphatase activity, obtained from dogs 10 to 15 days after ligation of the common bile duct. They found that the increased phosphatase activity in the blood of the recipients persisted for several days, falling to normal levels only after 5 to 7 days. The authors interpreted this slow disappearance of the injected phosphatase as indicating either that most of the normal
phosphatase of the bile does not come from the serum, or that the phosphatase in jaundiced blood is bound to some substance which retards its excretion by the hepatic cells. In similar experiments, Cantarow and Miller (1948) found that very little of the infused phosphatase could be recovered in the bile. The liver thus appears to play little part in clearing the injected alkaline phosphatase. Furthermore, Polin et al. (1962) presented positive evidence which indicates that the increased serum alkaline phosphatase in dogs after ligation of the hepatic duct is due to an increased production of the enzyme by the liver rather than decreased excretion.

Apparently contradicting results were obtained by LeVeen et al. (1950) who showed that calf duodenum alkaline phosphatase, injected intravenously into dogs, was removed rapidly from the circulation by the liver. The liver apparently excretes the injected material over a very long period, and the injected phosphatase appears in the bile at a constant rate, showing that a maximal secretory ceiling is reached. The full recovery of the injected dose in the bile indicates that the enzyme is not catabolized or inactivated and the liver serves primarily as a regulator of the secretion of this enzyme into the bile. It should be noted that the injected alkaline phosphatase used in this experiment came from a species of animal different from the recipient.

As will be evident from the preceding discussion, two opposing theories have been proposed regarding the increased serum alkaline phosphatase in obstructive liver disease. The first, the "retention" theory, states that the elevated phosphatase results from inability of
the damaged liver to excrete phosphatase made in other tissues, such as bone and intestine (Gutman, 1959). The second is the "regurgitation" theory which assumes that the liver does not excrete alkaline phosphatase, and that hyperphosphatasemia is due to regurgitation of the bile which contains alkaline phosphatase of hepatic origin (Polin et al., 1962). The results of Freeman and Chen (1938), Cantarow and Miller (1948) and of other workers (e.g. Sebesta et al., 1964; Kaplan, 1968; Posen, 1967) argue in favour of the regurgitation theory.

More recent work by Kaplan and Righetti (1970), on the origin and mechanism of elevation of rat serum alkaline phosphatase after bile duct obstruction, suggested that simple regurgitation of the liver phosphatase is only a partial explanation of the increased serum enzyme level, since concomitant induction in the liver phosphatase was essential for the serum elevation to occur. The increase in the liver alkaline phosphatase was several orders of magnitude greater than the amount of phosphatase that would normally be excreted in the bile and that hence would be available to accumulate in the liver following the bile duct obstruction. These authors also observed that the entire elevation in serum activity was due to a steady increase in a new isoenzyme that had the same properties as the liver alkaline phosphatase, and that the rise in serum activity was intimately related to de novo synthesis of this enzyme by the liver.

b) Other serum enzymes in hepatic disease

Increasing interest has been focussed on other serum enzymes both
as aids to the diagnosis of different liver diseases and to justify the interpretation of the elevated level of serum alkaline phosphatase. Measurements of serum alkaline phosphatase and 5'-nucleotidase could give a good indication of liver and bone disease (Sherlock, 1968). Moreover, the level of serum 5'-nucleotidase should be more specific for liver disease than bone disease, since there is relatively little 5'-nucleotidase in bone (Reis, 1951) as compared with liver (Essner et al., 1958). The subcellular distribution of 5'-nucleotidase in the liver is generally similar to that of alkaline phosphatase, activities being found in the plasma membrane lining the bile canaliculi and sinusoids with only a very small proportion of the activity in the cytosol (El-Aaser and Reid, 1969). An additional advantage of 5'-nucleotidase over alkaline phosphatase is that the former remains relatively constant in the same individual (Young, 1958).

Dixon and Purdom (1954) were the first to study the levels of 5'-nucleotidase in sera of patients with different diseases. They found elevated values in patients with disease of the biliary tree but normal values in those with neoplastic bone lesions. These authors were unable to find close correlations between 5'-nucleotidase levels and those of alkaline phosphatase or of other measures of hepatic function, in patients with obstructive jaundice or other forms of hepatobiliary disease. Since then, many workers have suggested that measurement of 5'-nucleotidase is a useful test in distinguishing liver from bone disease (e.g. Young, 1958; Hill and Sammons, 1967;
Batsakis et al., 1968; Lum and Gambino, 1972). Other investigators, on the other hand, have regarded the estimation of 5'-nucleotidase as inferior to that of alkaline phosphatase (e.g. Wachstein and Sigismondi, 1958; Eshchar et al., 1967; Davidge and Philpot, 1966) as a diagnostic aid.

Connell and Dinwoodie (1970) investigated the reliability of 5'-nucleotidase levels as a means of distinguishing liver from bone disease in humans, by comparing the activity of serum 5'-nucleotidase with the activities of the alkaline phosphatase isoenzymes. They used liver and bone alkaline phosphatases, extracted from the tissues of post-mortem humans, as markers for the isoenzymes on polyacrylamide gels. Particular interest was focussed on cases where the results of other biochemical tests on both tissues were equivocal. They reported that cases with normal liver function tests and raised 5'-nucleotidase were usually associated with increases in the liver alkaline phosphatase isoenzyme and clinical evidence of liver disease. On the other hand, cases with normal liver function tests and normal 5'-nucleotidase sometimes showed increases in both the bone and liver isoenzymes. In such situations, there was a very high incidence of carcinoma, mainly prostatic. They concluded that if routine liver function tests are already abnormal, further confirmation by 5'-nucleotidase estimation is unnecessary, but with normal liver function tests, an increased 5'-nucleotidase is a reasonable indication of the presence of some hepatic involvement.
While some studies showed that there is a difference in the behaviour of serum 5'-nucleotidase and alkaline phosphatase in the course of hepatic disease (e.g. Eshchar et al., 1967), others have confirmed a general high correlation in the level of the two serum enzymes (e.g. Batsakis et al., 1968). Hence the suggestion was made that the estimation of the two enzyme levels is of value in the differential diagnosis of liver disease (Phelan et al., 1971). These authors also confirmed the general high degree of correlation between abnormalities of serum alkaline phosphatase and 5'-nucleotidase in acute hepatobiliary disease. They represent the response of serum levels of these two enzymes in acute liver disease as reflecting the effects of factors which influence the production or release of both phosphatases essentially simultaneously. However, in chronic liver disease, the dissociation of the levels of the two enzymes suggested to them that changes may occur which operate selectively on the production or release of one or other enzyme at different stages of the illness.

Elevated levels of γ-glutamyl transpeptidase have been shown to be associated with liver disease (e.g. Lum and Gambino, 1972; Rutenburg et al., 1963). Moreover, Whitfield et al. (1972) confirmed a strong correlation between serum γ-glutamyl transpeptidase, alkaline phosphatase and 5'-nucleotidase in liver diseases. The diseases were grouped into two categories, those in which the lesion can be regarded as affecting principally the biliary system and its function, and those in which the disease causes mainly parenchymal cell damage. They
found that the average serum activities of all three enzymes were three to six times greater in biliary tract disease than in the parenchymal disease category. Hence, they concluded that for all three enzymes, changes in activity in serum reflect principally alteration in biliary function rather than damage to the parenchymal cells. The diagnostic advantage of \(\gamma\)-glutamyl transpeptidase, as reported by these authors, lies in its higher elevation relative to 5'-nucleotidase and alkaline phosphatase. Consequently they suggested that \(\gamma\)-glutamyl transpeptidase would be the enzyme of choice if only one estimation is to be relied upon for the detection of latent or chronic liver disease. However, there are two considerations against the use of this enzyme alone for the detection of liver disease. The first of these is the lack of specificity of this enzyme for hepato-biliary disease; the second is the possible induction of the enzyme in the liver by drug administration (barbiturates and phenytoin).

The mechanism of the rise in serum \(\gamma\)-glutamyl transpeptidase was investigated recently by Krysiewski et al. (1973) who found that, in rats, the peak levels of alkaline phosphatase and 5'-nucleotidase in serum were reached 24 h after ligation of the common bile duct, whereas, although the level of \(\gamma\)-glutamyl transpeptidase in serum rose rapidly, the peak level was not reached before 48 h and possibly even later. Moreover, if drugs known to inhibit protein synthesis are given to the rat immediately before ligation, the responses of the liver alkaline phosphatase, 5'-nucleotidase and \(\gamma\)-glutamyl transpeptidase are disparate. The administration of such drugs was found
to prevent the rise of serum and liver alkaline phosphatase, but did not affect the changes in 5'-nucleotidase and γ-glutamyl transpeptidase. Some alterations in the relative levels of the three enzymes were detected in the liver and serum after administration of such drugs to control animals. These results were enough to conclude that the early changes in serum activities of 5'-nucleotidase and γ-glutamyl transpeptidase do not appear to be due to a rapid increase in hepatic synthesis of these enzymes, but the elevated serum alkaline phosphatase activity was mainly due to the increased synthesis of this enzyme in the liver.

It seems then that during acute diseases affecting the biliary tract system, the three enzymes behave in similar, though not identical, ways while in chronic disease of this nature, measurements of 5'-nucleotidase and γ-glutamyl transpeptidase become a more sensitive test than alkaline phosphatase. However, the sensitivity of the serum alkaline phosphatase test and consequently its clinical reliability seems to depend largely on the substrate used in the assay medium. Generally persons using β-glycerophosphate as a substrate for alkaline phosphatase regard its estimation as superior to that of 5'-nucleotidase (e.g. Eshchar et al., 1967) in contrast to those using phenyl phosphate as a substrate (e.g. Lum and Gambino, 1972; Phelan et al., 1971). This topic will be considered in the Discussion section.

Serum leucine aminopeptidase (LAP) has also received considerable
attention from various clinical chemists and pathologists. Clinically, there is a general agreement that elevated levels of LAP (using L-leucyl-β-naphthylamide as substrate) in serum reveal the existence of diseases of the liver and hepatobiliary-pancreatic duct system (Szász, 1964; Batsakis et al., 1968; Mericas et al., 1964). But whether this enzyme can be used as an aid to differentiate between the various disorders that affect the liver, the bile duct or the pancreas is still a matter of question.

Rutenburg et al. (1958) found that patients with cancer of the pancreas not involving the hepatobiliary tract had normal serum LAP levels; but a significant increase was detected in many patients with cancer which did involve this tract. This test was also found to be a useful screening procedure for hepatobiliary disease in jaundiced and unjaundiced patients, but it does not distinguish between different causes of jaundice (Derrick et al., 1963; Mericas et al., 1964). It was stated by Bressler et al. (1960) that the assay of serum LAP adds no useful information not already provided by other tests in common use when differentiating between the various disorders that affect the hepatobiliary tract system. Pineda et al. (1960) reported that patients with carcinoma of the head of the pancreas or extrahepatic biliary tract had extremely high serum LAP activity. Activity was higher in patients with obstructive jaundice caused by tumours than in those with jaundice resulting from stones. Cancer of the tail or the body of the pancreas caused mild elevation of LAP activity in serum, and approximately the same elevation was found in patients with acute viral or drug-induced hepatitis. Serum LAP values of patients with common
duct obstruction were often elevated before bilirubin or even alkaline phosphatase. Consequently, Pineda et al. (1960) concluded that serum LAP assays were more sensitive than serum alkaline phosphatase in detection of hepatic metastasis.

Studies concerning the relative specificities of serum LAP, 5'-nucleotidase and alkaline phosphatase for obstructive disease of the pancreaticobiliary duct system are largely in agreement, with the exception of the work reported by Horký et al. (1967) and Wachstein and Sigismondi (1958) (see below). Kowllessar et al. (1961) submitted a lot of useful data on serum LAP activity and on the comparison of this enzyme with serum 5'-nucleotidase and alkaline phosphatase in the same patient. They reported that serum LAP is increased in diseases of pancreas and hepatobiliary tract characterized by either intrahepatic or extrahepatic obstruction of the biliary tree, or by infiltration or metastasis of the liver. Furthermore, they confirm the results of Rutenberg et al. (1958), in that serum LAP is normal in cases with carcinoma of the pancreas if obstruction of the common bile duct has not taken place. The view of Kowllessar et al. (1961) was later supported by Cutillo et al. (1961) and Szász (1964) who showed that the increase in serum LAP activity in hepatitis is not due to injury of the parenchyma, but to damage of the bile ducts. Elevation of LAP was found by Kowllessar et al. (1961) to be closely paralleled by rises in serum 5'-nucleotidase and alkaline phosphatase. Simultaneous determinations of alkaline phosphatase, LAP and 5'-nucleotidase were of value in determining whether the elevation of the first enzyme in
serum is due to liver or bone disease, a conclusion which was later confirmed by Batsakis et al. (1968) and Lum and Gambino (1972).

There are several enzymes in the body which will split the peptide link between leucine and another amino acid and may thus be called leucine aminopeptidases. Such enzymes are usually found not to be absolutely specific; they can also act on any N-terminal amino-acid with an aliphatic side-chain. Hill and Smith (1957) stated that "The nature of the side chain of the residue bearing the free α-amino group has strong influence on the rate of hydrolysis, whereas the residue bearing the nitrogen of the susceptible bond has little influence on the rate of hydrolysis". Since then, studies on the activity of aminopeptidase in mammalian systems have been directed primarily towards purification of this enzyme from tissue extracts in order to provide a definitive description of its chemical and physical properties. This work culminated in the isolation of aminopeptidases from pituitary, lung, spleen, thymus and kidney (Smith and Hill, 1960). The successful isolation and fractionation of the enzyme from liver and serum permitted the demonstration of a number of aminopeptidase isoenzymes (Behal et al., 1965). These isoenzymes show different behaviour patterns during hepatic disease, (Wachstein and Sigismondi, 1958; Horký et al., 1967). The aminopeptidase assay was more sensitive than any other biochemical index of biliary obstruction when using leucylglycylglycine and glycylglycine as substrates. Apparently the aminopeptidase activity measured with L-leucyl-β-naphthylamide as substrate does not
represent the total activity of this enzyme. Unfortunately, most workers in clinical chemistry and clinical pathology regarded the enzymes which catalyze the hydrolysis of L-leucyl-β-naphthylamide as being fully representative of the activity of leucine aminopeptidase (LAP; designated as Classical aminopeptidase).

Nachlas et al. (1962) evaluated the specificity of the aminopeptidases of a range of rat tissues using seven substrates, namely L-leucyl-, glycyl-, L-alanyl-, L-phenylalanyl-, L-methionyl-, L-glutamyl-, and L-arginy1-β-naphthylamides, in the whole tissue homogenates and in the partially purified enzyme. They demonstrated the non-specific nature of the aminopeptidase (LAP) as normally assayed with L-leucyl-β-naphthylamide. They also furnished strong evidence that during the purification procedure, another enzyme (s) is removed.

Furthermore, it seems evident that there are some differences between the aminopeptidases in normal serum and those associated with liver disease. By use of starch gel electrophoresis, Kowlessar et al. (1960) were able to demonstrate differences in the electrophoretic mobilities of the LAP activities of normal and jaundiced sera. Dioguardi et al. (1961) separated three peaks of LAP from normal serum using DEAE-cellulose chromatography. This pattern was severely altered when serum from patients with hepatobiliary disease was used. McDonald et al. (1964) were able to distinguish at least three aminopeptidases in blood serum. Only one of these is present in normal
serum, the other two appeared during tissue injury. The three enzymes could be distinguished by their thiol and cation dependence and by their substrate specificities. Further heterogeneity is indicated by the results of Behal et al. (1963) who chromatographically resolved the IAP activity of human serum into a number of aminopeptidase components. Seven peaks of aminopeptidase activity were recovered from DEAE-cellulose chromatography (Behal et al. 1964) designated as $\alpha_1$, $\alpha_2$, $\alpha_3$, $\beta_1$, $\beta_2$, $\beta_3$ and $\beta_4$. Only four of these aminopeptidase components ($\alpha_3$, $\beta_1$, $\beta_2$, $\beta_3$) also hydrolyze amino acid $\beta$-naphthylamide, at a rate comparable to that for the dipeptide.

However, on comparing the leucyl-$\beta$-naphthylamide hydrolysing enzyme found in liver with that in serum, it was found that enzymes from both tissues exhibit similarities in many respects. Both migrate to the $\alpha_1$-globulin region on cellulose acetate strips (Smith and Rutenberg, 1963), and show complete loss of activity with EDTA, which is restored by Co$^{++}$ and Zn$^{++}$ ions (Smith et al., 1965). Further evidence for this similarity was provided by the work of Behal et al. (1965) in their attempt to determine the tissue origin of the aminopeptidase components reported in serum (see above). As far as the liver in concerned, the aminopeptidase activity appears in two chromatographically distinguishable peaks designated as liver-$\alpha$-, and liver-$\beta$-aminopeptidases. The former showed maximum activity toward both leucylglycine and leucyl-$\beta$-naphthylamide with metal dependence. The latter has no activity on the dipeptide, no metal requirement and was sensitive to puromycin. By comparing the two
aminopeptidase components with that of serum they concluded that the liver may possibly be a source of serum $\alpha_3$-aminopeptidase, although the liver enzyme elutes somewhat ahead of the serum component (but does overlap to a certain extent). To them, it seems quite probable that the liver-$\beta$-aminopeptidase may be identical with part or all of the serum $\beta_2^-$, and $\beta_3$-aminopeptidases since all show similar elution profiles, divalent metal independence, resistance to inhibition by EDTA and puromycin sensitivity. As a result, the term 'arylamidase' (or naphthylamidase) has been used to designate more accurately the activity in the latter liver fraction (Behal et al., 1966).

The discussion in the above section is mainly restricted to the comparison of serum aminopeptidase with that found in liver, with special attention to clinical applications. Details of the purification methods, characterization and the structural studies are beyond the scope of this thesis. Such studies were nicely discussed by Stranes and Behal (1974) and Garner and Behal (1974).

Elevated levels of alkaline ribonuclease (RNase) have been reported in sera of a number of humans with cancer, (e.g. Migliarese, 1958; Levy and Rottino, 1960). Nevertheless, only a few systematic attempts to determine the usefulness of the assay in clinical oncology have appeared; perhaps because serum RNase elevations were not found in all studies of cancer patients. Studies have been reported showing that in 60% of cancer patients the average serum RNase level was significantly higher than the average level noted in a group of healthy subjects (Zytko and Cantero, 1963). These findings apparently
contradict the earlier results of Métails and Mandel (1955) and of Houck and Berman (1958), who reported no change in the serum RNase level in cancer patients. However, an increased serum RNase level was observed by Zigman and Allison (1959) in tumour-bearing rats, who suggested that this increase may be related to the tumour growth. On the other hand, feeding rats with diet containing 4-dimethyl-aminooazobenzene results in depression of serum RNase activity by 50-60% compared to that of control rats (Zytko and Cantero, 1963). They also found that in rats, serum RNase resembles "liver" RNase in its pH for optimal activity, but differs in that serum RNase is thermolabile and is completely destroyed by heating for 3 minutes in boiling water, while the liver enzyme is heat-resistant and shows only a loss of 15-20% of the activity under similar heating conditions (Zytko et al., 1958).

From the clinical data presented in this whole section, it would appear that serum 5'-nucleotidase, alkaline phosphatase, γ-glutamyl transpeptidase, and L-leucyl-β-naphthylamidase behave rather similarly in cases of liver disease. It will be rather interesting to know their tissue origin and if all are released by the same mechanism.
1 : 2) MORPHOLOGY OF THE LIVER

The liver, the largest organ in the body, is enclosed by the connective tissue capsule of Glisson and has a double blood supply. The portal vein brings venous blood from the intestines and spleen, and the hepatic artery, coming from the coeliac axis, supplies the liver with arterial blood. Both vessels, together with the lymphatic and bile ducts, enter the liver through the porta hepatis, inside which they divide into several branches to the right and left lobes. The terminal branches of the portal vein discharge their blood into the sinusoids (liver capillaries) which are irregularly disposed, normally in a direction perpendicular to the lines connecting the central veins. The direction of flow is determined by the higher pressure in the portal vein than in the central vein.

The liver is regarded as made up of single-cell plates or sheets, pervaded by two systems of tunnels, the hepatic and portal canals. The sinusoids are lined by large cells with only a very thin layer of cytoplasm. Some of these cells show remarkable phagocytotic activity. Although attempts have been made to distinguish different types of lining cell, little agreement has been reached (Aterman, 1963). The name Kupffer cell, which has been used for one of these types, is often extended to cover all sinusoidal lining cells. The rat liver differs from that of some other species in there being no demonstrable basement membrane to the sinusoids (Aterman, 1963). The lining cells
in the rat liver appear to be separated from the microvilli of the parenchymal cells solely by the space of Disse, which is filled by a loose network of reticulin fibres possibly embedded in a ground substance.

The excretory system of the liver begins with the bile canaliculi, which lie in grooves on the contact surfaces of the liver cells. The canaliculi networks drain into the thin-walled bile ducts and these terminate in larger ducts in the portal canals. The bile space is separated from the space of Disse by tight junctions between the liver cells, and generally there is no communications between these two spaces.

The interior of the liver cell contains numbers of distinct, membrane-bounded organelles. These are i) nucleus, ii) mitochondria, iii) endoplasmic reticulum, iv) lysosomes, v) Golgi apparatus, vi) peroxisomes, vii) plasma membrane. These systems of cell components may now be considered briefly.

The nucleus, the largest organelle of the cell, contains material which to the histologist is chromatin and to the biochemist deoxyribonucleoprotein. The application of electron microscopy to examine the nuclear structure allowed the description of a number of specialised regions in the nucleus. Such specialised regions are the nucleoli and the dispersed and condensed chromatin (Littau, et al., 1964; National Cancer Institute Monograph, 23, 1966). The nucleus has a double-contour membrane (Fawcett, 1955) with pores allowing
interchange with the surrounding hyaloplasm. The outer membrane is continuous with the endoplasmic reticulum (Watson, 1955) and has attached ribosomes (Palade, 1955). The nucleus was found to be the storage site of the genetic information. It is also believed to contain some of the energy metabolism enzymes (Georgiev, 1967) and to synthesise ribonucleic acid, some of which is transported from the nucleus to the cytoplasm as ribonucleoprotein particles. In the cytoplasm, such particles form ribosomes and polysomes which are found either free or attached to the endoplasmic reticulum membranes.

The mitochondria are large organelles which are surrounded by a double membrane, the inner membrane being invaginated to form grooves or cristae and differing from the outer membrane in enzymology, lipid composition and density (Schnaitman et al., 1967; Parson and Yano, 1967). Both membranes show the usual three-layered structure and have the same dimensions (Thompson et al., 1968). Within these two membranes, an enormous number of energy-providing processes take place (e.g. Roodyn, 1967).

The endoplasmic reticulum appears as lamellar profiles; if it is lined by granules, called ribosomes, it is designated rough endoplasmic reticulum, this is involved in the synthesis of 'export' protein. Endoplasmic reticulum without ribosomes is smooth and is the site of a variety of enzymes including those responsible for the detoxification of drugs and synthesis of steroids. The lysosomes are dense bodies, adjacent to the bile canaliculi. They contain many hydrolytic enzymes which, if released, as they might be under pathological circumstances,
could destroy the cell. The Golgi apparatus consists of a system of particles and vesicles, again lying near the canaliculus. The aspect of function of the Golgi-endoplasmic reticulum-lysosome (GERL) complex will be discussed later in connection with the fate of animal glycoprotein. The peroxisomes are the microbodies long known to the electron microscopists to be of similar size to lysosomes. They are hard to separate from lysosomes, but differ from them in having a dense core of osmophilic material. They contain oxidoreductases rather than hydrolases (Roodyn, 1967). The plasma membrane, the seventh part of the liver cell will be discussed in detail in the following section.
1:3) THE PLASMA MEMBRANE CONCEPT

The surface layer surrounding living cells, which acts as a barrier between the interior of the cell and its external milieu, is called the plasma membrane (Smith, 1962).* Broadly speaking, two main functions can be attributed to the plasma membrane: it plays a role in intracellular metabolism; and mediates interactions between the cell and its external environment.* The simplest of these interactions maintains a desirable intracellular milieu by bringing in needed substances and getting rid of waste products. The plasma membrane acts as a passive diffusion barrier to charged and large molecules, and in addition carries out facilitated diffusion, active transport, endocytosis (pinocytosis and phagocytosis), and exocytosis.

On a more complex level, cells must communicate with each other; this is especially important in the formation and maintenance of multicellular organisms. The plasma membrane functions in the secretion and reception of hormones, the conduction of nerve impulses, and in direct cellular interactions such as adhesion and contact inhibition.

Finally, on yet another level, the plasma membrane may be involved in such sophisticated processes as immunological defence and information storage. The concept of plasma membrane has been recently reviewed by DePierre and Karnovsky (1973).

* Detailed studies of plasma membrane function, which is intimately

* Synonym: cell membrane.
connected with the membrane structure, demanded the isolation of such membrane in a pure state, essentially free of intracellular membranes.

Isolation of plasma membrane may conveniently be divided into five sequential steps: choice of the tissue to be used, selection of markers, disruption of the tissue, fractionation and analysis. Some valuable information has come from investigations on erythrocyte plasma membrane using erythrocyte ghosts (e.g. Whittam, 1962; Glynn, 1968). Mouse and rat livers were the next most popular tissue chosen for plasma membrane isolation in the vast majority of cases (e.g. Emmelot and Bos, 1962; Neville, 1960; Evans, 1969; Hinton et al., 1970). Plasma membranes from many other tissues have also been studied.

In any isolation of one component from a mixture, it is obviously necessary to have an assay for that component. Assays for the other components present in the original mixture are also of critical importance. Moreover, to follow the distribution of a certain organelle, two approaches can be used. First, if the organelle in question has a distinctive morphology, microscopy can be used to evaluate purity. Secondly, characteristic enzymes may be used to follow the distribution of an organelle throughout the fractionation process.

As far as liver plasma membrane is concerned, both morphological and enzymatic markers are fortunately available. Liver cells form junctional complexes with neighbouring cells (Bloom and Fawcett, 1968).
These junctions are easily identified in electron micrographs and appear to remain intact during homogenisation of the liver. Membrane sheets were obtained lying adjacent to such complexes or stretching between two junctions (Neville, 1960). The microvilli extending into the bile canaliculi were also found to be resistant to gentle homogenisation (Emmelot et al., 1964). These features provide excellent morphological criteria for the identification of liver cell plasma membrane. Furthermore, Benedetti and Emmelot (1968a,b) described other distinctive features of the appearance of rat liver plasma membrane. The plasma membrane is different from the endoplasmic reticulum in being thicker and in showing a triple-layer structure more readily. The hexagonal appearance of the plasma membrane surface is shared by neither the endoplasmic reticulum nor the mitochondria. Finally the globular knobs, revealed by negative staining, are different from those on the endoplasmic reticulum and mitochondrial membrane. However, the problem of how to quantify the morphological markers arises. It is difficult to achieve random sampling and to decide exactly what to quantify, especially on a minute scale as that used for electron microscopy, though some progress has been made in this field (de Duve, 1971).

In spite of all the problems associated with the use of electron microscopy to elucidate plasma membrane structure, this technique nevertheless does permit general agreement that the plasma membrane is made up of a lipoprotein core covered by a peripheral glycoprotein coat, and contains on its surface specialised junctional complexes.
High magnification electron micrographs of plasma membrane showed the presence of a triple-layered structure with an average width of about 80 Å. However, the total width of the membrane element and the dimensions of the individual layers may vary from cell to cell. In addition, different fixatives appeared to reveal different dimensions (Benedetti and Emmelot, 1968a). The protein and the polar groups of the phospholipids contribute to the heavily stained strata, whereas the light layer may represent lipid. The width of the light zone is such as to accommodate a lipid bilayer (Robertson, 1964; Sjöstrand, 1968) thus providing an experimental verification for the bileaflet hypothesis of Danielli and Davson (1935). The extracellular side of the membrane was found to be chemically different from the inside, since different chemical reactivities were observed to fixing agents. Such asymmetry is not restricted to a differential affinity of the two membrane leaflets for fixing and staining agents: certain enzymes (Whittam and Ager, 1964), ionogenic groups (Wallach et al., 1966), insoluble antigens and sialic acid (Benedetti and Emmelot, 1968a) have also been demonstrated to be asymmetrically located.

There is now a good deal of evidence that a glycoprotein or a polysaccharide-rich layer coats the surface of many, if not all, mammalian cells (e.g. Benedetti and Emmelot, 1968b). This glycoprotein coat is probably filamentous in nature and responsible for most of the surface properties of the cell. Benedetti and Emmelot (1965) showed a globular structure on the plasma membrane surface after negative staining with phosphotungstate. These globular units are of an average diameter of 50–60 Å and attached to the membrane
either directly or via a short constriction of about 20 Å. Such a granular appearance was also demonstrated by Cunningham and Crane (1966) on the outside surface of the intestinal microvilli. Accordingly the plasma membrane was claimed to be identifiable in a mixture containing different membrane types. Isolation of the granulated particles from different tissues (Oda and Seki, 1966; Emmelot et al., 1968) supports the view that such particles represent a specialised region of the cell surface and are not artifacts of the staining method. These globular areas seem to be enzymatically highly distinguishable from the other parts of the plasma membrane (Emmelot and Visser, 1971, see p.39).

Although it seems that the whole plasma membrane has the same fundamental structure, a number of specialised regions were found on the cell surface, viz. the junctional complexes. Three types of junctions have been described: the desmosome, the intermediate and the tight junctions. In an endothelium, the tight junction acts as a continuous belt surrounding a segment of the cell, separating the intracellular space from the lumen (Farquhar and Palade, 1965). Under the electron microscope, these junctions appeared to have a five-layered structure originating from two membranes (two triple-layer) fused together. No glycoprotein could be detected in the tight junctions between epithelial cells (Rambourg, 1966; Rambourg and Leblond, 1967), perhaps because the carbohydrate coat of the outer membrane leaflets is removed when the two membrane are fused. The continuity of the central line was found to be interrupted after the tight junctions had
been treated with EDTA. After such treatment, the intermediate line appeared under the electron microscope as a row of globules rather than as the continuous dense line found in the untreated junction (Benedetti and Emmelot, 1968a).

The intermediate junction and desmosome are each built up from two plasma membranes cemented together on the extracellular side. Both junctions are believed to act as attachment devices. The intermediate junction is characterised by an intercellular plug of relatively low density and by a thick layer of cytoplasmic material surrounding it from either side. The width of these junctions was found to be different in different cells; indeed they may be completely absent. Such changes in the width are compensated by changes in the width of the tight junctions (Farquhar and Palade, 1963). Desmosomes, which are small patches on the cell's surface, are characterised in thin sections by a thick, dense intracellular plug with a laminar appearance. The cytoplasmic layer appears as a short, circumscribed plaque rather than as a diffused thick layer (Benedetti and Emmelot, 1968a).

It is worth pointing out here that isolated fragments of plasma membrane from different portions of the cell will have quite distinct morphological structures. Hence, it is risky to rely only on morphology to test the purity of the isolated plasma membrane.

The other means by which the plasma membrane can be identified is by the use of marker enzymes, but these must be validated by
cytochemical staining. These techniques have their own difficulties, but the plasma membrane localization for a number of enzymes has been successfully proved. Briefly, the classical enzyme markers used for plasma membrane were:

- **5'-nucleotidase** (e.g. El-Aaser et al., 1970; Fleischer and Fleischer, 1969; Evans, 1969; Hinton et al., 1970; Giacobino and Perrelet, 1971; Reid, 1967; Coleman et al., 1967; Wolff and Jones, 1971).
- **Leucine aminopeptidase** (e.g. Evans, 1969, 1970a; Hübscher et al., 1965).
- **Adenyl cyclase** (e.g. McKeel and Jarett, 1970; Wolff and Jones, 1971; Pohl et al., 1971; Ray, 1970).
- **Alkaline phosphatase** (e.g. Emmelot et al., 1964; Graham et al., 1968).
- **(Na\(^+\)/K\(^+\)) ATPase** (e.g. Gahmberg and Simons, 1970; Yamashita and Field, 1970; Evans, 1970a; Wallach and Kamat, 1964).

From the above discussion, it seems that a combination of morphological markers and enzyme activities might provide excellent criteria for the purity of isolated plasma membrane fragments.
In any cell fractionation technique, the aim of the procedure is to separate a particular cell component intact and undamaged. In the case of liver cell plasma membrane, this is impossible, since this membrane must be broken in order to release the contents of the cell and to obtain plasma membrane free from contamination. So far, the integrity of the cell-membrane has been sacrificed in all the isolation procedures. What is also of importance, is the extent to which the membrane is fragmented throughout the homogenisation process. Mild mechanical homogenisation (e.g. the Potter-Elvehjem homogeniser; Hinton, 1972) breaks the cell-membrane into large sheets sedimenting with the nuclear fraction and small fragments sedimenting in the microsomal fraction. In case of drastic rupture of the cell (e.g. gas cavitation, Kamat and Wallach, 1965; or pressure cell treatment, Cook et al., 1965), the membrane becomes fragmented to vesicles which sediment with the microsomal fraction.

Methods have long been available for purification of plasma membrane sheets from the crude nuclear fraction. A nice review of the purification of large sheets of plasma membrane was presented by Hinton (1972).

In brief, Neville (1960) was the first to purify rat liver plasma
membrane. His technique consisted of a gentle mechanical homogenisation of the liver cells in bicarbonate buffer. The large sheets of plasma membrane were mainly concentrated in the slow speed pellet (1500 x g for 10 minutes). Contaminating materials were removed by multiple washing and finally the plasma membrane was purified by isopycnic flotation in a discontinuous sucrose gradient at a density of 1.16 - 1.22. Since then, this procedure has been modified by introducing other sucrose solutions of different densities in the final flotation step in order to reduce the contamination by mitochondrial membranes (e.g. Emmelot et al., 1964; Touster et al., 1970). With the development of the zonal centrifugation technique, many investigators (e.g. El-Aaser et al., 1966a; Evans, 1969, 1970a, b; Hinton et al., 1970; Prospero and Hinton, 1973), took advantage of the large volumes of zonal rotors to compensate for the low yield of the initial technique.

According to the above mentioned authors, the plasma membrane isolated from the crude nuclear fraction (whether from zonal rotors or isopycnic flotation in tubes) originates predominantly from the bile canalicicular side of the cell-membrane. With the isolated plasma membrane sheets, the existence of small vesicles was also reported (e.g. Pfleger et al., 1968; Benedetti and Emmelot, 1968a). Apart from this microscopic detection of some vesicles Evans (1970b) was the only one who reported that the plasma membrane isolated from the crude nuclear fraction does contain subfractions of different density and enzymatic composition. Since the results of Evans are closely
related to our results, these will be discussed in detail together with the results obtained during this investigation.

As the plasma membrane isolated from the crude nuclear fraction appeared to be derived mainly from the bile canaliculi, the sinusoidal membrane was expected to be concentrated in the microsomal fraction (Hinton et al., 1971). Kamat and Wallach, (1965) were the first to attempt the separation of the microsomal plasma membrane from the bulk of the microsomes (i.e. the endoplasmic reticulum fragments) of Ehrlich ascites carcinoma cells. They subfractionated microsomes by isopycnic centrifugation on Ficoll gradients and found the plasma membrane at a median density of 1.07. Graham et al. (1968) used essentially the same procedure to separate the plasma membrane fragments which sedimented with the microsomes of rat liver. From their results it appeared that the plasma membrane so prepared was very little contaminated with other fractions as far as the enzyme activities reported can tell. Erecińska et al. (1969) also claimed to have isolated rat liver microsomal plasma membrane by the method of Kamat and Wallach but no further details were given.

House and Weidemann (1970) separated the microsomal plasma membrane using a discontinuous Ficoll gradient of densities 1.104, 1.055 and 1.036. The plasma membrane, collected in the 1.036 Ficoll density band was found to be rich in 5'-nucleotidase, phosphodiesterase and ATPase as compared to the whole liver homogenate. Touster et al. (1970) used a discontinuous sucrose density gradient buffered with 5 mM Tris-HCl, pH 8.0, as an alternative procedure for preparation of
microsomal plasma membrane. They recovered the plasma membrane as a turbid white material of density 1.15 with relative specific activities of 5'-nucleotidase and phosphodiesterase about 20 times that of glucose 6-phosphatase.

Advantage has also been taken of zonal centrifugation rotors in the microsomal subfractionation technique. El-Aaser et al. (1966b) were able to perform an isopycnic zonal centrifugation on rat liver postmitochondrial supernatant in a B-IV zonal rotor. Fractionation was carried out by an isopycnic sedimentation procedure using a shallow sucrose gradient of density 1.05 to 1.2. They found that, with no metal ions added to the homogenisation medium or gradient, separation did occur between the low density elements bearing 5'-nucleotidase and those with glucose 6-phosphatase activity, but there was a considerable overlapping between the two peaks. Addition of 4-15 mM Mg$^{2+}$ ions was reported to improve the separation by virtue of a shift of the glucose 6-phosphatase-containing vesicles to a higher median density.

Hinton et al. (1971) attempted to separate the plasma membrane (of microsomal size) from the endoplasmic reticulum using an exponential sucrose gradient in B-XV and B-XIV zonal rotors. They reported that in the absence of Mg$^{2+}$ ions, only the rough endoplasmic reticulum fragments are obtained in purified form, with very little separation between the peak activities of 5'-nucleotidase and glucose 6-phosphatase in the light zone of the gradient. The best separation
between 5'-nucleotidase and glucose 6-phosphatase was obtained only after the addition of 5 mM Mg\(^{2+}\) to the homogenisation medium and the gradient, but the purification of the plasma membrane fragments did not rise as much as they had expected. Flotation rather than sedimentation resulted in better separation between the two enzyme peaks. Treatment of the microsomal fraction, after sonication, with 0.5 mM Pb\(^{2+}\) resulted in the isolation of a fraction highly enriched with fragments containing 5'-nucleotidase activity. Moreover, Hinton et al. (1971) reported that after lead treatment the distribution of the plasma membrane markers, other than 5'-nucleotidase, indicates that the membrane fragment responsible for the nucleotidase activity peak is not fully representative of the plasma membrane material. Such treatment resulted in the separation of types of plasma membrane fragments with distinctive differences in enzyme content.

Thines-Sempoux et al. (1969) subfractionated the microsomal fraction resuspended in 0.25 M sucrose, 3 mM imidazole, in the Beaufay rotor (Beaufay, 1966; specially designed for isopycnic sedimentation), with a sucrose density gradient extending to 1.34. They studied the distribution of the endoplasmic reticulum enzymes together with some of the plasma membrane marker enzymes. They were able to detect a group of vesicles enriched with 5'-nucleotidase and cholesterol with a low median density as compared to that of smooth and rough endoplasmic reticulum. A similar distribution was found for the outer mitochondrion membrane fragments as indicated by monoamine oxidase activity. These results, together with those of
other investigators describing the heterogenous distribution of the plasma membrane marker enzymes in the microsomal fraction will be further discussed together with our own results.
As has been described, the cell membrane is a complicated structure consisting of a lipoprotein membrane coated with a glycoprotein-rich layer. The application of zonal centrifugation for large scale isolation of purified plasma membrane facilitates the accumulation of knowledge about the morphological structure and enzymatic composition of this liver fraction. However, relatively little is known about the antigenic structure of liver cell plasma membrane. Liver cell-specific antigens have long been demonstrated to be mainly associated with the membrane system of the cell (Dumonde, 1966). This membrane system involves the endoplasmic reticulum, which is continuous with the outer nuclear membrane on one hand and the plasma membrane on the other hand (Robertson, 1960).

Studies on the isolated plasma membrane revealed that the antigenic activity is mainly restricted to the outer layer of this membrane (Benedetti and Emmelot, 1968a).

Emmelot et al. (1964) were the first who attempted immunological characterization of rat liver plasma membrane. They reported that anti-(plasma membrane), prepared against the isolated rat liver plasma membrane, yielded four precipitation lines when tested against the isolated membrane or whole liver homogenate, by the Ouchterlony double diffusion or the immunoelectrophoresis technique. They
suggested that, in order to obtain such results, the four membrane
antigens must be soluble in the physiological saline present in the
agar, so that antigens tightly bound to the membrane would not be
detected. Extraction of plasma membrane with physiological saline
removed about 26% of the membrane proteins, in which the following
antigens were detected: one rat-serum component, one antigen shared
by other tissues and two liver-specific antigens. Anti-(plasma
membrane), raised against the physiological saline-insoluble membrane
proteins, showed only one precipitation line when tested against the
saline-soluble material. Hence, Emmelot et al. (1964) concluded
that this antigen is intrinsically present in the saline-insoluble
membrane core, which, they say, may be equated to the plasma
membranes as they appear in situ. The saline-soluble proteins are,
at least partly, a preparative artifact representing non-membraneous
protein. Neville (1968) reported that treatment of the isolated liver
plasma membrane with 0.05 M sodium carbonate removed a mixture of
proteins; one of these proteins was found to be a liver-specific antigen.
The absence of more details about this specific antigen makes further
discussion rather difficult.

Sheffield and Emmelot (1972) studied the nature of the tissue-
specific antigens in the liver cell surface and on the isolated plasma
membrane. They raised antibodies against the isolated liver plasma
membrane washed with 0.15 M- NaCl to remove proteins adsorbed on
to the membranes during the isolation procedure (Emmelot et al., 1964).
Such an anti-(plasma membrane) was found to be not absolutely specific
for liver plasma membrane. Sheffield and Emmelot (1972) reported that absorption of the anti-(plasma membrane) with a mixture of rat serum, liver homogenate supernatant and kidney powder removed all the non-specific antibodies. By application of immunofluorescence techniques, the pre-absorbed antiserum was demonstrated to be specifically staining the isolated plasma membrane and to outline the cells in frozen sections. Specially localised staining was reported to be at the bile canaliculi and space of Disse. Moreover, Sheffield and Emmelot (1972) classified the antigenic activities of the liver cell surface into three groups:

1 - Antigens associated with the globular knobs on the extracellular side of the plasma membrane.

2 - Antigens associated with the membrane.

3 - Masked antigens.

1 - Antigens associated with the globular knobs

The existence of globular knobs on the surface of the plasma membrane lining the bile canaliculi and space of Disse was shown by electron microscopy (Benedetti and Emmelot, 1968a,b). Treatment of the isolated membrane with papain resulted in the removal of these globular structures and the release of almost all the aminopeptidase activity from the membrane (Emmelot and Visser, 1971). Moreover, treatment of the liver cells with such proteases caused the loss of about three quarters of the original antigenic activity of the cell surface (Sheffield and Emmelot, 1972). Hence
the conclusion was drawn that most of the antigenic activity of the cell surface resides in the papain-released particles which are associated with the aminopeptidase activity. The loss of antibody reaction from the bile space membrane and the space of Disse, after absorption of the anti-(plasma membrane) with the papain-released particles, confirmed the localisation of the particles in this part of the cell surface.

Fractionation of the total papain digest by passing it through a Sephadex G-200 column resulted in the separation of high and low molecular weight fractions (Emmelot and Visser, 1971). Moreover, absorption of the anti-(plasma membrane) with the high molecular weight fraction removed all the antibodies responsible for staining the bile space membrane. By this procedure Sheffield and Emmelot (1972) presented evidence that the low molecular weight particles are those coating the Disse space, whereas the high molecular weight fraction is mainly located in the bile space membrane.

2 - Antigens associated with the membrane

These antigens account for the immunofluorescence which remains on the membrane area after removal of the globular knobs from the cell surface, by proteases.

3 - Masked Antigens

During the course of treatment of the liver cells or the isolated plasma membrane with proteases, the antigenic sites of the membrane
showed a dual response. Complete digestion of the membrane with the proteases resulted in the removal of all the globular knobs. Trypsin and papain each caused a significant transient increase in the antigenic activity of the cell surface and that of the isolated plasma membrane. Such an increase was interpreted by Sheffield and Emmelot (1972) to be due to the exposure of new antigenic sites on the cell surface. The masking substances were found to be proteins with basic and/or aromatic side groups. Such masking phenomena were previously reported to occur on some cell surfaces, the masked antigens being exposed after alteration of the cell surface either by viral transformation or by enzymatic treatment (Burger, 1969; Häyry and Defendi, 1970).

New antigenic sites on the isolated liver plasma membrane have also been reported to be exposed after treating the isolated membrane with alkali buffer, pH 10.2 (Gurd et al., 1972). A $^{125}$I-labelled anti-(mouse liver plasma membrane) was raised against the 0.15 M-NaCl-washed mouse liver plasma membrane. Relative to the binding capacity of the unfractionated plasma membrane, Gurd et al. (1972) reported that extraction with alkali buffer removed 60-70% of the antigenic activity, while the alkali-insoluble fraction possessed slightly increased binding capacity compared to the untreated membrane. Hence they suggested that the removal of the alkali-soluble components resulted in the exposure of additional antigenic determinants that were previously masked. Moreover, these authors reported that treatment of the alkali-insoluble fraction with 33% aqueous pyridine solubilized
about 33% of the membrane proteins. The ability of the pyridine-
soluble and pyridine-insoluble fractions to bind to the anti-(plasma 
membrane) indicated that the proteins in those fractions retained 
their native antigenic structure. From the extent of cross-reactivity 
between the alkali-soluble, pyridine-soluble and pyridine-insoluble 
fractions, these authors concluded that the alkali-soluble antigens 
are normally exposed on the cell surface, while the pyridine-soluble 
antigens are buried within the membrane.

Gurd et al., (1973) investigated in more detail the proteins 
obtained from solubilizing mouse liver plasma membrane by alkali 
and aqueous pyridine. They reported the presence of six antigens 
in the alkali-soluble fraction which account for approximately two-
thirds of the antigenic activity of the intact membrane as reported by 
Gurd et al. (1972). One of these antigens is probably a glycoprotein 
with an approximate molecular weight of 120,000. Furthermore, 
they suggested that the increase in the apparent antigenic activity of 
the membrane after extraction with alkali could be attributable to 
increased activities of two of the antigenic determinants of the liver 
membrane. These authors also reported that treatment of the 0.15 M-
NaCl-washed plasma membrane with detergent (1% deoxycholate + 
1% Triton X-100) solubilizes about 50% of the membrane proteins. 
Two dimensional immunoelectrophoresis of the detergent extract against 
anti-(plasma membrane) indicated the presence of six antigens. Taking 
all the results of these authors together, it appears that none of the 
antigens is selectively extracted by the alkali nor by the pyridine
Blomberg and Perlmann (1971a) made a detailed examination of a detergent extract of isolated rat liver plasma membrane. They reported that 70% of the isolated membrane protein is solubilized with 1% sodium deoxycholate + 0.5% Lubrol W (cetylpolyoxyethylene condensate). Immunoelectrophoresis of the detergent extract against anti-(plasma membrane) antiserum indicated the presence of at least eighteen antigenically active components. By histochemical staining, some of these antigens were found to be associated with enzymic activities (Blomberg and Perlmann, 1971b). Two 5'-nucleotidase isoenzymes were demonstrated to be associated with two negatively charged proteins of the same electrophoretic mobilities. Six precipitation lines had both nucleoside diphosphatase and triphosphatase activities. These antigenic proteins with dual enzymatic activities were interpreted as due to the presence of an apyrase activity in the liver membrane. Moreover, Blomberg and Perlmann (1971b) detected a Mg\(^{2+}\)-independent ATPase in the solubilized plasma membrane fraction which is different from the Mg\(^{2+}\)-dependent ATPase reported by Emmelot and Bos (1966). This Mg\(^{2+}\)-independent ATPase is apparently similar to that reported by Wattiaux-deConinck and Wattiaux (1969). However, plasma membranes used by Blomberg and Perlmann (1971a,b) were not washed with 0.15 M-NaCl to remove non-membraneous proteins (Emmelot et al., 1964). Hence one must be cautious in interpreting these immunoelectrophoretic histochemical studies, particularly the demonstration of an antigen with catalase
activity, and six positively charged antigenic proteins with no enzyme activity.

Recently, Blomberg and Raftell (1974), using plasma membrane washed with 0.15 M-NaCl, were able to detect twelve antigenically active proteins in the 1% deoxycholate + 0.5% Lubrol W extract by a two-dimensional immunoelectrophoresis technique. They resolved the six lines of nucleoside di- and triphosphatase activities reported by Blomberg and Perlmann (1971b) into at least ten different precipitates with the enzyme activity. In addition, two of these lines were reported to hydrolyse L-leucyl-β-naphthylamide. The authors explain these results by the solubilization of a multienzyme complex present in rat liver plasma membrane.

Sheffield and Emmelot (1972) drew attention to the fact that even the 0.15 M-NaCl-washed plasma membranes contain antigenic components which are common to other subcellular fractions of the liver or to serum. This was demonstrated by cross-reacting rat serum or liver cytosol with anti-(plasma membrane) raised against plasma membrane washed with 0.15 M-NaCl. Blomberg and Perlmann (1971a) showed that at least one antigen with an esterase activity is common between rat liver plasma membrane and liver microsomal fraction. This observation was recently confirmed by Blomberg and Raftell (1974) using 0.15 M-NaCl-washed plasma membrane. The latter authors investigated in more detail the common antigens between these two liver fractions. The plasma membrane and the microsomal fraction share three antigenic proteins with nucleoside di- and triphosphatase
activities. L-Leucyl-β-naphthylamide hydrolysing enzyme was reported to be associated with two of these three antigens when plasma membrane was tested against anti-(microsomal fraction). The exact identities of L-leucyl-β-naphthylamidase-active antigens in microsomes and those of the plasma membrane were not well established. Apparently contradicting results were reported by Gurd et al. (1973) showing that there was no cross-reaction between the anti-(mouse liver plasma membrane) and other subcellular fractions or even between the anti-(mouse plasma membrane) and mouse serum (Gurd et al., 1972). Apart from the work of Gurd and co-workers, other results indicate that the plasma membrane may contain proteins common to other subcellular fractions and serum; alternatively the proteins may be simply artifacts of the isolation procedure. The answer to this problem, may help in elucidating the relation between plasma membrane of the liver cells and the blood.
1 : 6) BIOSYNTHESIS AND SECRETION OF GLYCOPROTEINS

The term "glycoprotein" has been defined by Gottschalk (1966) as "Conjugated proteins containing as prosthetic group one or more heterosaccharides with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the polypeptide chain". These conjugated proteins are represented by many substances of biological importance including enzymes, hormones, antibodies and membranes. The polypeptide components of glycoproteins are synthesized in relation with ribosomes of the endoplasmic reticulum (Redman and Cherian, 1972). After their completion, the carbohydrate side chains are added (Spiro, 1969) in a stepwise manner to the polypeptide chains as they migrate through the endoplasmic reticulum - Golgi complex system (Schachter et al. 1970). The carbohydrate content of the glycoproteins may vary from less than one per cent to more than eighty per cent of the weight of the molecule, and as few as two to as many as seven sugar types may be present in a given protein. The monosaccharide components of mammalian glycoprotein are synthesized by way of nucleotide sugars. The most commonly found monosaccharides in glycoproteins are: D-galactose, D-mannose, L-fucose, D-glucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and the various derivatives of neuraminic acid (the sialic acids) (Spiro, 1969). Other monosaccharides have been found in trace amounts in some glycoproteins.
It is well known that most serum proteins contain carbohydrate, and the oligosaccharide chains in these glycoproteins are thought to be branched (Wagh et al., 1969). The liver has been shown to be the major site of synthesis of serum glycoprotein (Macbeth et al., 1965). In the liver, the carbohydrate moieties of the secretory glycoproteins are added in a defined sequence to the nonreducing termini of the growing polypeptide chains as monosaccharide units, catalyzed by a multi-enzyme system, designated a multiglycosyltransferase system. Schachter et al. (1970) reported that some of these transferase enzymes, namely N-acetylgalactosaminyl-, galactosyl-, and sialyl-transferases are located in the Golgi apparatus of rat liver and therefore, it may be involved in secretion of glycoproteins from the liver into the blood stream.

Fucose has long been detected in most of the plasma glycoproteins (Winzler, 1960) and in mammalian tissue glycoproteins (Foster and Ginsburg, 1961). These monosaccharide residues were reported to be located at the ends of the carbohydrate side chains of the glycoprotein (Spiro, 1969). The uptake of fucose may indicate completion of the synthesis of these side chains and presumably of the glycoprotein molecules themselves. Hence, an understanding of the subcellular organelle at which fucose is added to the carbohydrate side chain may elucidate further the mechanism for the biosynthesis and secretion of glycoprotein by the liver. Fucose is
a stable monosaccharide and is selectively taken up into glycoproteins via its nucleotide derivative (Foster and Ginsburg, 1961). Furthermore, these authors reported that fucose is not extensively (less than 2%) metabolized in the Krebs cycle or to other glycolytic intermediates. These results encouraged the use of radioactively labelled fucose as a precursor of glycoproteins.

Bennett and Leblond (1970) reported that the columnar cell of duodenal villi in rats is exceptionally able to utilize labelled fucose. Glycoproteins containing labelled fucose were detected at 2.5 minutes after injection in the Golgi apparatus only. The percentage labelling of this subcellular fraction decreased with time, while labelling of the cell surface started to appear after 5 min. and continued to increase with time. This finding suggested to them that uptake of fucose for completion of glycoprotein takes place at the Golgi apparatus. The completed glycoprotein is then rapidly transferred to the outer surface of the plasma membrane to be added to the "cell coat". They found that some radioactivity is associated with vesicles in the rest of the cell, these were suggested to be carriers of glycoprotein from the Golgi complex to the cell coat. The vesicles were reported later to be the cell lysosomes taking up the labelled fucose slightly before any significant labelling is observed at the cell surface (Bennett and Leblond, 1971). This indicates that lysosomes receive their newly completed glycoprotein directly from the Golgi apparatus. These authors also reported that the Golgi apparatus of rat liver cells is the main site of incorporation of fucose,
as in columnar cells. The Golgi complex also acts as a site of completion of synthesis for lysosomal glycoprotein.

Sturgess et al. (1973) investigated in more detail the incorporation of fucose in the rat liver cell and they confirmed that the Golgi complex is the major site of the uptake. They demonstrated that fucose is first incorporated in the cisternae of the Golgi apparatus, then passes to the network of fine tubules which surround the cisternae and finally to the secretory vesicles. With the disappearance of labelled fucose from the Golgi complex, the radioactivity was mainly localized over the plasma membrane and its associated smooth-surfaced vesicles. These results suggested that glycoproteins were exported in vesicles to the plasma membrane for secretion from the liver cell. The correlations between the fall in radioactivity of the Golgi apparatus, the appearance of radioactivity on the plasma membrane and the corresponding rise in activity of serum glycoprotein suggested that the glycoprotein synthesized in the liver is released at the plasma membrane directly into the blood. Riordan et al. (1974) focussed their attention upon the stage of glycoprotein biosynthesis between the Golgi apparatus and plasma membrane. They found that the peak specific radioactivity of the Golgi apparatus was at 15 min. after injection; the specific activity then declined to a minimum at 1 hr. In the case of the plasma membrane, there was an early rapid rise in specific activity, which peaked at 10 min., then declined until 30 min., and finally rose slowly between 30 min. and one hour. They also reported that when the specific activity in the plasma membrane rose
rapidly, that of serum rose slowly, followed by a more rapid increase between 15 and 30 min., at which time the specific activity of the plasma membrane decreased. These results suggested to them that two distinctive fractions exhibiting two different rates of turnover of fucose are reaching the plasma membrane. The first is a rapidly turned-over fraction which probably represents the secretory glycoprotein; the second is the less rapidly turned-over fraction and probably represents glycoproteins to be added to the plasma membrane coat. Moreover, these authors claimed that this mechanism for secretion of glycoproteins is also a representative one for the secretion of proteins from the liver, unlike that reported by Franke et al. (1971).

Franke et al. (1971), using L-(guanido-$^{14}$C) arginine, reported two different pathways by which proteins would move from the rough endoplasmic reticulum to the plasma membrane. The first is from the rough to the smooth endoplasmic reticulum to the Golgi apparatus by which the proteins are packaged into secretory vesicles; these vesicles then break off from the Golgi apparatus and migrate toward the plasma membrane. The second pathway is from the rough to the smooth endoplasmic reticulum from which the proteins migrate in the form of vesicles toward the plasma membrane. This mechanism was conjectured from finding that the peak specific radioactivity in the endoplasmic reticulum is at 10 min. after injection. The specific activity then declines rapidly between 10 and 20 min., and gradually thereafter. Membrane proteins of the Golgi apparatus were labelled
more slowly, with a peak specific activity at 30 min. The specific radioactivity of the plasma membrane protein was reported by these authors to have two distinctive peaks. The first was at about 20 min. and the second at about 60 min. after injection. These results suggested to them that it is not necessary for the newly synthesized protein for secretion to pass through the Golgi apparatus.

Although it is uncertain whether the material reaching the plasma membrane for secretion follows the mechanism postulated by Franke et al. (1971) or that suggested by Riordan et al. (1974), the material reaching the extracellular part of the plasma membrane must either be released immediately or temporarily form part of the cell coat and then be released into the extracellular fluid.

The investigation presented in this thesis is therefore intended to elucidate the mechanism of release of material from the plasma membrane into the circulating fluid, which may help to understand the biological role of the liver cell plasma membrane.
THE AIMS OF THIS STUDY

When the work described in this thesis was begun, many workers had already investigated the correlations between the levels of certain liver and serum enzymes during the course of hepatic disease. We became interested in this problem because a number of enzymic indicators of liver disease, notably 5'-nucleotidase, alkaline phosphatase and to some extent L-leucyl-β-naphthylamidase are located in the hepatocyte plasma membrane. The main objective of this study, therefore, has been to determine whether or not the plasma membrane is the source of these enzymes in blood. The proposed approach to this problem has been the application of immunological techniques to investigate the antigens common to rat serum and rat liver plasma membrane.

The liver cell plasma membrane is a heterogeneous structure which consists of two major functionally and morphologically distinctive areas, the bile-facing area and the sinusoidal-facing area. After homogenisation, the plasma membrane of the liver cell breaks down into large sheets recovered with the nuclear fraction and small vesicles mainly concentrated in the microsomal fraction. A method for isolating highly purified plasma membrane sheets had already been developed in this laboratory. The author therefore intended to attack the problem of detecting the antigens common to rat serum and liver cell plasma membrane by using isolated plasma membrane sheets and fluorescein.
labelled anti-(rat serum). If there are indeed such antigens, then
affinity chromatography of rat serum on immobilised anti-(plasma
membrane) would result in the isolation of the serum proteins that are
homologous to those of plasma membrane. To elucidate the nature of
any common antigens, the protein fraction would be examined for the
presence of carbohydrate and other constituents and for enzymic activity.

As mentioned above, the vesicles derived from the plasma
membrane are concentrated in the microsomal fraction. It had for some
time been suspected in our laboratory that the microsomal plasma
membrane may originate from the sinusoidal-facing surface of the liver
cell which contribute part of the serum proteins. As the function and
morphology of this part of the plasma membrane is different from that
of the isolated sheets, it is of interest to determine whether or not the
two are enzymatically and immunologically identical. This type of
study necessitated isolation of the plasma membrane vesicles in a pure
state. It is only recently that methods have been developed for
separating plasma membrane vesicles from the microsomal fraction.
Little work had been published describing the heterogeneity of the
marker enzymes within the population of plasma membrane vesicles,
or the possibility that these vesicles may derive largely from the
sinusoidal surface of the cell. It was desirable, therefore, to examine
the distribution of plasma membrane marker enzymes among the plasma
membrane elements of microsomal size. It was also hoped to apply an
in vivo labelling technique to examine the nuclear and microsomal
fractions for sinusoidal surface fragments.
CHAPTER TWO

MATERIALS AND METHODS
2:1) MATERIALS

In general, analytical grade chemicals, obtained from either BDH Ltd., Hopkins and Williams Ltd. or Koch-Light Ltd. were used.

Tris buffer [tris-(hydroxymethyl)-aminoethane] and Norit A activated charcoal were bought from Sigma Chemical Co. Ltd. Lubrol W (cetylpolyoxyethylene condensate; now called CIRRASOL ALN-WF) was a gift from I.C.I. Sepharose-4B was obtained from Pharmacia Ltd. (U.K.). Freund complete adjuvant was bought from DIFCO Lab. (East Molesey, U.K.). Mineral water sucrose was obtained from Tate and Lyle Ltd. L-^{14}C-Fucose was purchased from the Radiochemical Centre, Amersham (U.K.). Anti-(rat serum) was obtained from Wellcome Reagent or as globulin fraction from Mercia Ltd. (U.K.) and Ionagar No.2 used was from Oxoid Ltd. (U.K.).

Fluorescence measurements were done on a Perkin Elmer MPF-3 spectrophotometer. Radioactivity measurements were performed in a Packard 3003 Scintillation Counter using Lissapol-scintillant unless otherwise stated (see section 2:5-d). All immunoelectrophoreses were carried out in Shandon electrophoresis apparatus. Polyacrylamide gel electrophoresis was performed with a home-made, Shandon type, apparatus using 9 cm x 5 mm glass tubes.

All centrifugation was carried out at 4° in M.S.E. centrifuges. Table 2-1 provides a summary of the type of rotors employed for various separations. Further details of particular centrifugation technique are given below.
<table>
<thead>
<tr>
<th>Centrifuge Rotor</th>
<th>Fraction separated</th>
<th>$g$-applied</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor Swingout</td>
<td>Nuclear pellet</td>
<td>$600 \times g_{\text{max}}$</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>Pellet of floated material from the whole homogenate (see B XIV)</td>
<td>$600 \times g_{\text{max}}$</td>
<td>10 min</td>
</tr>
<tr>
<td>Mistral 6L Swingout</td>
<td>Serum</td>
<td>$2000 \times g_{\text{max}}$</td>
<td>20 min</td>
</tr>
<tr>
<td></td>
<td>To remove precipitated protein</td>
<td>$2000 \times g_{\text{max}}$</td>
<td>~10 min</td>
</tr>
<tr>
<td></td>
<td>(does not have to be accurate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A XII</td>
<td>Plasma membrane sheets</td>
<td>$800 \times g$ (Sample Zone)</td>
<td>50 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2500 \times g$ (End of gradient)</td>
<td></td>
</tr>
<tr>
<td>High Speed 18</td>
<td>Mitochondria and Lysosomes</td>
<td>$11500 \times g_{\text{max}}$</td>
<td>15 min</td>
</tr>
<tr>
<td>Super Speed 65</td>
<td>Floated plasma membrane</td>
<td>$135000 \times g_{\text{max}}$</td>
<td>90 min</td>
</tr>
<tr>
<td></td>
<td>Plasma membrane pellet</td>
<td>$135000 \times g_{\text{max}}$</td>
<td>90 min</td>
</tr>
<tr>
<td></td>
<td>Microsomal pellet</td>
<td>$135000 \times g_{\text{max}}$</td>
<td>60 min</td>
</tr>
<tr>
<td></td>
<td>Washing and solubilization of plasma membrane</td>
<td>$135000 \times g_{\text{max}}$</td>
<td>90 min</td>
</tr>
<tr>
<td>B XIV</td>
<td>Floated material from whole liver homogenate (Centre)</td>
<td>$42000 \times g$</td>
<td>90 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$98000 \times g$ (Edge)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasma membrane fragments from Microsomal pellet (Centre)</td>
<td>$52000 \times g$</td>
<td>16 hour</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$120000 \times g$ (Edge)</td>
<td></td>
</tr>
</tbody>
</table>
a) Animals

The animals used were hooded rats of the University of Surrey strain. The original breeding nucleus was obtained by the University from the Specific Pathogen-Free Unit, Allington Farm, Ministry of Defence, Porton Down, Salisbury. Male animals about 6-7 weeks old were used for all experiments.

Six rabbits were used to raise the anti-(liver plasma membrane) antiserum (three New Zealand White and three Dutch). Anti-(liver cell cytosol) antiserum was raised in three Dutch rabbits.

All animals are supplied by the University of Surrey Animal Unit and housed under conditions of constant temperature (20°C), and lighting on a 12 hour light/dark cycle (0.6.30-18.30 light phase).

b) Tissue preparation

Rat livers were the only tissue used. After killing the animal by cervical dislocation, the tissue was perfused with warm 0.25 M sucrose solution, buffered with either 5 mM sodium bicarbonate, pH 7.8, or 5 mM Tris-HCl, pH 8.0. Perfusion was done by pumping in the solution through a needle inserted in the aorta or the portal vein (anaesthetized rat), after slashing the inferior vena cava just above the diaphragm.

The tissue was extracted rapidly and put into ice-cold 0.25 M sucrose. After cooling, the tissue was weighed wet, minced with scissors and homogenised in ice-cold 0.25 M sucrose. To remove the connective tissue, the homogenate was filtered through a coarse sieve. Detailed description of the exact homogenisation conditions will be left to the appropriate section.
Whenever possible, all assays were carried out on fresh samples. However, it is imperative that the assays of glucose 6-phosphatase (G.6.Pase) be performed as soon as possible on the fractions. The more stable estimations, e.g. alkaline phosphatase, phosphodiesterase, alkaline ribonuclease, nucleic acid and protein which are not affected by storage, were assayed within 3-4 days. Samples were stored at -23°C. All enzyme incubations were carried out in a shaking water bath at 37°C.

a) Protein estimation

Protein was measured by the Autoanalyser adaptation (Schuel and Schuel, 1968) of the method of Lowry et al., (1951) as it was modified by Hinton and Norris (1972). The "Low sensitivity" manifold described by Hinton and Norris (1972) was used to measure the protein content of the zonal AXII fractions, with Bovine serum albumin (BSA) standards ranging from 0.1-1 mg/ml. The "high sensitivity" manifold was used for measurements of the protein of all other fractions (with BSA standards of 0.01-1 mg/ml).

b) Enzymes releasing inorganic phosphate (Phosphatases)

The following phosphate releasing enzymes (phosphatases) were assayed: 5'-nucleotidase
Glucose 6-phosphatase
Acid β-glycerophosphatase
Adenosine diphosphatase (ADP)
Adenosine triphosphatase (ATP)
All the phosphatase estimations were performed as reported by Prospero et al. (1973). In general the assay system contained:

- 0.5 ml tissue sample, 0.4 ml buffer, and 0.1 ml substrate. Assays on reference samples (homogenate, nuclear fraction, floated material from the whole homogenate and microsomal fraction) were normally done in duplicate. The contents of the assay tubes were thoroughly mixed with Whirlimixer and incubated at 37° for a period of time within which the enzyme reaction was linear (Hinton, 1970). Due to the presence of some endogenous phosphate, substrate and tissue blanks were always included. Tissue blanks were performed, for every single fraction, by adding 0.1 ml distilled water instead of the substrate. Substrate blanks were assayed, normally in duplicate, using 0.5 ml distilled water in place of the tissue sample.

The reaction was stopped by adding 1.5 ml of 6% trichloroacetic acid and after a period at 4°, the precipitated material was spun down. The supernatant was assayed for total inorganic phosphate content by an automated method (Hinton et al., 1970) adapted from the method of Lowry and Lopez (1946). As standards, sodium dihydrogen orthophosphate solutions (0.1-1.5 μmoles PO₄³⁻/ml) were used. The standards were treated as the samples, i.e. 1.5 ml trichloroacetic acid was added per 1 ml of the standard.

c) p-Nitrophenol releasing enzymes

Alkaline phosphatase and alkaline phosphodiesterase were the only p-nitrophenol releasing enzymes assayed throughout this study. The estimations of these two enzymes were made by the automated method of Hinton and Norris (1972).
d) **L-Leucyl-β-naphthylamidase**

The method employed was that of Goldbarg and Rutenburg (1958) as modified by Hubscher et al. (1965). The assay mixture contained 0.5 ml tissue sample, 0.25 ml of 0.2 M phosphate buffer, pH 7.0, and 0.25 ml of 1.06 mg/ml solution of L-leucyl-β-naphthylamide dihydrochloride. After incubation, the solution was deproteinised by the addition of 0.5 ml of 6% trichloroacetic acid, and the precipitated proteins were spun down. The β-naphthylamine released by the enzyme remains in the supernatant and was assayed using an automated method adapted from that of Norris (1973) with β-naphthylamine standard solutions (2-30 μg/ml) to which 0.5 ml of the stopping solution was added per 1 ml of standard.

A modification to the manifold described by Norris (1973) was made to correct the air to liquid ratio and to cut down the amount of reagents consumed. Flow rates were set so as to allow for the correct time delays between the addition of various reagents. Standards were used to check the performance of manifold (Fig. 2-1) and the sensitivity was found to be within 2% of the values obtained by Norris (1973).
### Autoanalyzer Manifold Used for the Estimation of β-naphthylamine

The solutions used were:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₂</td>
<td>0.1% aqueous</td>
</tr>
<tr>
<td>Ammonium Sulphamate</td>
<td>0.05% aqueous</td>
</tr>
<tr>
<td>NNED, N(-1-naphthyl)-ethylenediamine</td>
<td>0.05% in 96%乙醇</td>
</tr>
<tr>
<td>Sample Wash</td>
<td>0.4% perchloric acid</td>
</tr>
</tbody>
</table>

**Sampling rate:** 30 samples per hour
**Colorimetry at 561 nm with 1.0 cm flow cell**
**DC, double mixing coil (Technicon Catalogue No. 105-87)**
e) Succinate dehydrogenase

Succinate dehydrogenase was measured by the method of Pennington (1961). To a 0.5 ml aliquot of tissue was added 0.25 ml of a 1.5 mg/ml solution of INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride] in 0.5 M phosphate buffer, pH 7.4. The reaction was started by the addition of 0.25 ml of 0.3 M sodium succinate, pH 7.4, and after incubation for 10-20 minutes the reaction was stopped by 1.5 ml of 6% trichloroacetic acid. The red formazan was extracted into 4 ml ethyl acetate which was then separated from the aqueous phase by a brief spin. The colour of the extracts was measured at 490 nm against ethyl acetate blank. Tissue blanks were determined by substituting the succinate with 0.3 M sodium malonate, pH 7.4, in the assay medium.

f) Alkaline ribonuclease

This enzyme was measured essentially as described by de Duve et al. (1955). The assay mixture consisted of 0.5 ml tissue fraction, 0.3 ml of 0.25 M Tris-HCl, pH 7.8, buffer; 0.1 ml MgCl₂ (50 mM; or 50 mM EDTA) and 0.1 ml of 10 mg/ml RNA (Grade B torula yeast, Calbiochem). After incubation for about 1 hr at 37°, the reaction was stopped by 1.5 ml of 10% perchloric acid/0.25% uranyl acetate which precipitates all unreacted RNA and the large oligonucleotides. The supernatant contains oligonucleotides with a chain of less than about six nucleotides (Burge, 1973). To account for endogenous nucleotides and other soluble material which might absorb at 260 nm, it was
necessary to obtain a tissue blank reading for every sample. Samples
and substrate blanks were assayed in duplicate. Tissue blanks and
reagent blanks were set up exactly as above but with the addition of
the stopping reagent immediately after RNA being added. All tubes
were left in ice for 30 minutes prior to removal of the precipitate by
centrifugation. The supernatant was decanted, diluted with an equal
volume of distilled water and measured for absorbance at 260 nm.

g) *Estimation of Nucleic Acid*

The procedure described by Fleck and Begg (1965) was followed.
In detail, perchloric acid was added to a 5 ml tissue sample to give a
final concentration of 0.2 N and, after leaving for 10 minutes at 4°,
the precipitate was collected by centrifugation. If necessary 0.2 to
0.4 mg of bovine serum albumin were added as a carrier. The precipitate
was washed twice with 0.2 N perchloric acid and then extracted at 37°
with 4 ml of 0.3 N KOH. After 1 hr, 2.5 ml of 1.2 N perchloric acid
were added, the tubes cooled to 0°, and the precipitate spun down.
This was washed with 1.5 ml of 0.2 N perchloric acid, the washings
combined with the extracts and left overnight to allow all the potassium
perchlorate to crystallise. The extinctions at 230 and 260 nm were
measured and the concentration of RNA calculated by use of the formula
given by Fleck and Begg (1965).

h) *Catalase determination*

This enzyme was assayed by the automated method of Leighton
*et al.* (1968) adapted from the method of Chantrenne (1955) as
modified by Baudhuin et al. (1964). It is worth mentioning here that the solution of TiOSO$_4$ prepared as described by Leighton et al. (1968) was found to be very turbid, and consequently inaccurate results were obtained. Therefore, a more dilute solution was used; other reagents used were exactly as those described by Leighton et al. (1968) (Fig. 2-2). Standards of hydrogen peroxide solution were used to check the validity of the assay using the new concentration of TiOSO$_4$. The results were found to be as good as those reported by Leighton et al. (1968).
Fig 2-2

Automated catalase assay.
The solutions used were:

Triton, 1% Triton X-100 + 0.33% bovine serum albumin in 2% NaCl.
Substrate, 0.07% of 30% H₂O₂ in 0.033M imidazole- HCl buffer, pH 7.0
Washing fluid, 0.05% of BRIJ-35 (Technicon) in water
TiOSO₄, a stock solution prepared by mixing 82.5 ml of 15% TiSO₄ in sulphuric acid. (BDH) and 43 ml conc. sulphuric acid. The solution made up to one litre with distilled water and boiled for 30 minutes, cooled and filtered through Whatman no. 44 filter paper. The solution loaded into the autoanalyser was 16 times dilution of the stock solution.

Sampling rate, 20 samples per hour.
Colorimetry at 402 nm with 1.0 cm flow cell.
a) Preparation of Density Gradients

Sucrose gradients were used in all the experiments with zonal rotors described in this thesis. Sucrose solutions were prepared from a stock 2 M sucrose solution and the concentrations were checked by measuring their refractive indices at 20° with an Abbé refractometer (Bellingham and Stanley, Ltd., London). Traces of impurities, including proteins, were removed from the stock solution by stirring with Norit A activated charcoal (50 g/Kg sucrose) for about 30 minutes (Steele and Busch, 1967). The charcoal was removed by filtration through Whatman no. 54 filter paper. Refiltration normally was required before a clear solution was obtained.

Two types of a "home-made" gradient-maker were employed:

i) an "exponential" gradient maker, used in all experiments with the A-XII zonal rotor.  

ii) With the B-XIV zonal rotor, the more flexible gradient maker essentially described by Hinton and Dobrota (1969) was employed.

(i) Preparation of exponential gradient

The set-up used for the preparation of this type of gradient is shown in Fig. 2-3a. Initially 250 ml of 0.3 M sucrose (density 1.038 g/ml) were placed in the mixing vessel mounted on a magnetic stirrer. The following sucrose solutions were allowed to flow sequentially from the reservoir as the gradient was extracted from the mixing vessel.

600 ml of 1.164 M (density = 1.171 gm/ml)
500 ml of 1.656 M (density = 1.219 gm/ml)
a) Diagrammatic representation of the exponential gradient maker.
b) Diagrammatic representation of the double pump gradient maker used for the preparation of linear sucrose gradient.
Since the system is air-light, the volume in the mixing vessel remains constant at constant pressure. Consequently, the solutions in the reservoir flow in at the same rate as that at which the gradient was extracted.

(ii) Preparation of linear density gradient

The gradient maker used for preparation of this type of gradient is shown in Fig. 2-3b. The pump used was a double channel one with adjustable flow rates (Metering Pump Ltd.) giving flow rates of 0-50 ml/min. The mixing vessel, mounted on a magnetic stirrer, initially contained 360 ml of 0.6 M sucrose (density = 1.077 gm/ml). Liquid was pumped out from the mixing vessel into the zonal rotor at 40 ml/min. while 2 M sucrose was being added at 20 ml/min, until the whole solution from the mixing vessel was extracted. The density of the resulting gradient solution was found to be ranging from 1.077 - 1.257 gm/ml.

b) Loading and Unloading of the Gradients

The gradients prepared as above (linear or exponential) were loaded, light end first, to the outside of the zonal rotor. Normally the refractive index of the gradient fed into the rotor was recorded as a check of the shape of the gradient. When the exponential gradient had been loaded into the A XII zonal rotor, the remaining space was completely filled with 2 M sucrose as a 'cushion'. The sample was then injected carefully through the central line of the feed head, the 'cushion' being displaced from the edge of the rotor. An overlay, 0.08 M sucrose, was added to displace the sample away from the core.
The central line was then attached to a reservoir of the overlay solution to allow for the expansion of the rotor. The lead to the outside of the rotor was clamped and the rotor then accelerated from the loading speed (about 400 revs/min.) to 3700 revs/min.

The B-XIV rotor was used to subfractionate microsomes by flotation. Hence, the sample, adjusted to a density higher than that of the densest part of the gradient, was pumped to the outside of the rotor at the end of the linear gradient. Finally the rotor was filled completely with 2 M sucrose as a 'cushion'. During loading of the gradient and sample the rotor was spinning at about 2000 revs/min. then it was accelerated to 45000 revs/min.

After centrifugation for the required period of time the rotor was decelerated to the loading speed and the contents of the rotor was displaced by pumping in, through the edge line, 2 M sucrose. The set-up used for loading and unloading the zonal rotors was essentially as that described by Dobrota (1971).
a) **Isolation of Plasma Membrane Sheets**

In early experiments rat liver plasma membrane sheets sedimented with the nuclear pellet were separated by the procedure of Hinton et al. (1970). The nuclear fraction (see Table 2-1), obtained from perfused livers homogenised in 0.25 M sucrose, was loaded immediately on to the A XII zonal rotor in front of the exponential gradient prepared as described in Section 2:4a(i). Later the procedure developed by Prospero and Hinton (1973, "second procedure") in the separation of the hepatoma plasma membrane was used. Such procedure allows for more material to be processed in a single centrifugation by introducing a flotation step prior to the zonal centrifugation, as outlined in Fig.2-4. In detail, 50-60 gm of perfused livers were homogenised in 0.25 M sucrose containing 5 mM NaHCO₃, using three strokes of the Potter-Elvehjem homogeniser with the pestle rotated at 900 revs/min. The clearance between pestle and vessel was 13/1000 in. The homogenate was then filtered and the density adjusted to 1.19 gm/ml (ref. index 1.4037) with 2 M sucrose. Normally sufficient medium was used to give a homogenate containing 100-150 mg liver per ml. After taking out an aliquot, as a reference sample, the material was loaded on to the B XIV zonal rotor, 200 ml of 0.25 M sucrose was added as an overlay and the rotor finally filled with 2 M sucrose. During loading of sample and sucrose solutions, the rotor was spinning at 2000 revs/min, it was then accelerated to 40,000 revs/min and kept at this speed for 90 min (for g values applied see Table 2-1).
After centrifugation, 2 M sucrose was used to pump out the contents of the rotor as described in section 2:4b. The overlay (150 ml) was discarded and about 150 ml of the floated material, at the interface between the sample layer and the overlay, was then collected. This material was diluted with an equal volume of ice-cooled distilled water and spun down on a bench centrifuge (10 min at 600 x g). The pellet was resuspended in 50 ml of the homogenisation medium using a hand-operated Teflon/glass homogeniser (Jencons Ltd., Hemel Hempstead, U.K.). An aliquot was removed for various assays, and the suspension was loaded on to the zonal A XII rotor containing an exponential gradient buffer with 5 mM bicarbonate, identical to that described above. After spinning at 3700 revs/min for 50 min (Table 2-1), the rotor was decelerated and unloaded as described in section 2:4b and 40 ml fractions were collected. The tubes containing the plasma membrane fragments as judged either by the absorbance at 650 nm or by assaying 5'-nucleotidase were combined. The density of the pooled fractions was adjusted, with 2 M sucrose, to 1.19 gm/ml (ref. index 1.4037) and rehomogenised using three strokes of the Potter-Elvehjem homogeniser with the pestle rotating at 2400 revs/min. A 30 ml aliquot of the suspension was placed in each of the eight transparent centrifuge tubes, overlaid with 15 ml of 0.25 M sucrose and spun at 40,000 revs/min (Fig. 2-4). The plasma membrane fraction which was found at the interface of the overlay and the sample layer was collected, diluted with an equal volume of ice-cold distilled water and centrifuged at 40,000 revs/min for 90 min. The plasma membrane pellets were either resuspended in physiological phosphate buffered saline (section 2:8) or washed as described in section 2:6.
PERFUSED LIVER
homogenised, 3 strokes
900 revs/min

Density 1.19 gm/ml; B XIV Rotor, overlay with 0.25 M sucrose
40,000 revs/min
90 min

FLOATED MATERIAL
Dil. with dist. water
600 x g
10 min

PELLET
Resuspend in 0.25 M sucrose
10 strokes, hand homogeniser
2 gm/ml

A XII ROTOR
3700 revs/min, 50 min

PM FRACTION
Density 1.19 gm/ml; homogenised,
3 strokes, 2400 revs/min

FLOAT at 40,000 revs/min, 90 min
Remove pellicles

Dil. with dist. water
40,000 revs/min
90 min

PLASMA MEMBRANE
PELLET

Fig. 2-4: Outline of the two procedures used to separate Plasma membrane sheets
b) Fractionation of Liver Microsomes

Rat liver microsomes were subfractionated by flotation using a linear sucrose gradient in a B XIV zonal rotor. In these experiments rats livers were perfused with warm (\(~37\)°) 0.25 M sucrose containing 5 mM Tris-HCl buffer, pH 8.0, and homogenised in the same medium (cold) using three strokes of the Potter-Elvehjem homogeniser with the pestle rotated at 900 revs/min. The clearance between pestle and vessel was 13/1000 in. The concentration of the homogenate, after filtration, was brought to about 8 ml per gram of liver. The homogenate was then centrifuged at 10,000 revs/min for 15 min (Table 2-1) to remove particles larger than microsomes. The supernatant was then spun for 60 min at 40,000 revs/min to pellet the microsomes. The pellet was resuspended in 35 ml of 2 M sucrose containing 5 mM Tris-HCl, pH 8.0, by using two strokes of the Potter-Elvehjem homogeniser with a pestle rotated at 2400 revs/min. After taking out an aliquot, the suspension was loaded under a linear sucrose gradient ranging in density from 1.077 to 1.257 g/ml prepared as described in section 2:4a. The gradient was buffered with 5 mM Tris-HCl, pH 8.0. The rotor was finally filled with 2 M sucrose. After centrifugation for 16 hours at 45,000 revs/min (Table 2-1), the contents of the rotor were displaced with 2 M sucrose, and 20 ml fractions were collected. Details of the loading procedure and displacement of the gradient can be found in section 2:4b.

c) SITS Labelling of the Sinusoidal Surface

SITS (4-Acetamido-4'-iso-thiocyanato-Stilbene-2,2'-disulphonic acid disodium salt; BDH) is a compound known to bind to protein and
keep its fluorescence property. The use of this compound in various studies of the permeability of red blood cells has been reported (e.g. Knauf and Rothstein, 1971). Consequently, in the present study the fluorescence of the SITS-protein complex was used as an aid for the morphological identification of the sinusoidal surface and to follow its distribution in the plasma membrane fragments separated as described in section 2:5a and b.

The livers of rats anaesthetized with ether, were perfused through the portal vein with warm, about 37°, 0.25 M sucrose, 5 mM Tris-HCl, pH 8.0, until completely blanched. Each was then perfused with approximately 30 ml of a warm and freshly prepared solution of 50 μM SITS in 0.25 M sucrose, pH 8.0. A further perfusion with 0.25 M sucrose ensured that the unbound SITS was completely washed out of the liver. Small pieces were taken from different parts of the liver and were either fixed with formalin, or kept in ice-cold 0.25 M sucrose for morphological studies.

The livers from two rats (about 20 gm) were mixed and homogenised in ice-cold 0.25 M sucrose, pH 8.0, as described earlier. After filtration through a coarse sieve the volume was adjusted to 8 ml per gram of liver. The homogenate was fractionated by differential pelleting into a crude nuclear fraction, a mitochondrial + lysosomal fraction and a microsomal fraction (Table 2-1). The crude nuclear fraction was resuspended in 40 ml of 0.25 M sucrose, pH 8.0, and then fractionated as described in section 2:5a above. The microsomal fraction was processed exactly as described in section 2:5b above.

After centrifugation, the fractions obtained were digested in 14%
KOH. To assure complete digestion, incubation at 45° was carried out either overnight or for 2 days, and in some instances sonication was necessary to obtain complete solubilization of all membranes. Fluorescence at 483 nm with excitation at 350 nm (Knauf and Rothstein, 1971) was measured on the digests and on reference samples.

d) L-\(^{14}\)C-Fucose labelling of glycoprotein

An attempt was made to follow the incorporation of L-\(^{14}\)C-fucose into glycoprotein of different plasma membrane fragments isolated by the above-mentioned procedures. Furthermore, to differentiate between the secretory glycoprotein synthesised in the liver and that permanently associated with the plasma membrane fragments, the following experiment was designed.

Male rats (200-250 gm) were anaesthetized and injected intravenously with L-\(^{14}\)C-fucose (10 μCi/rat). After 15 and 90 minutes of injection, the rats were exsanguinated and the livers were perfused as fast as possible via the aorta with warm 0.25 M sucrose, 5 mM Tris-HCl, pH 8.0. The livers were fractionated by zonal centrifugation as described in section 2 : 5c. Radioactivity in the fractions collected, after displacing the zonal rotors, was measured as follows: 0.5 ml of the fractions, pipetted into scintillation vials, was made up to 1 ml with distilled water; 10 ml of the Lissapol-Scintillant (Table 2-2) was added and the vials were counted in a Packard Scintillation Counter.

Serum was separated from the pooled blood and fractionated by affinity chromatography on an immobilised anti-(plasma membrane) as described in section 2 : 10'. An aliquot of the serum was removed for
immuneelectrophoresis and to separate the carbohydrate-rich glycoprotein by following the method of Sturgess et al. (1972). An equal volume of cold 1.2 N perchloric acid was added to the fraction and centrifuged at 1800 revs/min for 20 minutes. The pellet which contained the perchloric acid-insoluble material was extracted with chloroform/methanol/ether 2:1:1, centrifuged as before and the supernatant discarded. The pellet was dried and dissolved by incubation with 0.5 N NaOH for up to 2 days at 45° and counted for radioactivity using the Tritosol-Scintillant (Table 2-2).

The supernatant which contained the perchloric acid-soluble fraction (seromucoid) was precipitated with 1/5 volume of 5% phosphotungstic acid in 2 N HCl. After standing in ice for 20 minutes, the precipitate was centrifuged, washed with 5% trichloroacetic acid and centrifuged. The pellet was washed with chloroform/methanol/ether, and counted as described in the previous paragraph.

<table>
<thead>
<tr>
<th>Lissapol-Scintillant</th>
<th>Tritosol-Scintillant</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 parts Toluene</td>
<td>106 ml Ethanol</td>
</tr>
<tr>
<td>1 part Lissapol</td>
<td>37 ml Ethylene glycol</td>
</tr>
<tr>
<td>0.4% PPO</td>
<td>600 ml Xylene</td>
</tr>
<tr>
<td>0.03% Dimethyl POPOP</td>
<td>257 ml Triton X-100</td>
</tr>
<tr>
<td></td>
<td>3 gm PPO</td>
</tr>
</tbody>
</table>

Table 2-2: Scintillation mixture used in the study
The pellets obtained after spinning the floated material (see section 2:5a) or the pooled fractions from the B XIV rotor (see section 2:5d) were extracted sequentially with 0.15 M-NaCl (Sheffield and Emmelet, 1972) and 0.2 M-bicarbonate, pH 9.0 (Weihing et al., 1972) successively. The bicarbonate buffer was prepared as 0.1 M sodium carbonate - 0.1 M bicarbonate, brought to pH 9.0 with NaOH. The membranes were resuspended in 5 ml of the washing medium using 10 strokes of the Jencons hand-operated homogeniser. The homogeniser was rinsed with another 5 ml, and the combined solution was left overnight at 4°C. The membrane was then pelleted by centrifugation at 40,000 revs/min for 90 minutes and the pellets rinsed by resuspending in 10 ml of the washing medium and repelleting immediately. The washed membrane pellets were either resuspended in an appropriate volume of 0.15 M-NaCl, or re-homogenised in 1% sodium deoxycholate + 0.5% Lubrol W using three strokes of the Potter-Elvehjem homogeniser rotating at 2400 revs/min. Detergent extraction was carried out at 4°C overnight. Undissolved material was then removed by centrifugation at 40,000 revs/min for 90 minutes. The supernatant was collected and the pellet resuspended in the same extraction medium.

For immunoelectrophoresis and immunodiffusion (see section 2:11), salt and detergent extracts were concentrated in a "Diaflo" ultrafiltration apparatus (Amicon Ltd., U.K.) using a PM-10 membrane, followed by an Amicon B 15 concentrator, to give the required protein concentration.
Antibodies against rat liver plasma membrane were raised in six rabbits; three rabbits were used to raise antibodies against rat liver cell cytosol. The cytosol used as an antigen was prepared from perfused liver, as the supernatant after pelleting the microsomes (Table 2-1). The plasma membrane used for immunisation was that extracted by 0.15 M-NaCl and bicarbonate as described in the previous section. This procedure should have removed traces of non-membrane protein which might adhere to the plasma membrane during the isolation procedure. Both antisera were raised by injecting the rabbits intramuscularly at two weeks intervals with 1.5 ml of the appropriate suspension. Such suspensions were made up of 2 ml of 6 mg/ml solution of plasma membrane or cytosol in physiological phosphate-buffered saline (0.15 M-NaCl/0.01 M phosphate, pH 7.5) (PBS) and Freund complete adjuvant (1:2). Each rabbit received four injections and after four weeks another injection at the same protein concentration was given. In order to keep the antibody titres of the antisera high, the rabbits were injected at four-weekly intervals with half the protein concentration mentioned above.

When antisera were required, the rabbits were bled at the 8th day after boosting. The serum was separated (see Table 2-1) and the globulin fraction was precipitated by adding, whilst stirring, an equal volume of saturated ammonium sulphate solution, and leaving the mixture overnight at 4°C before collecting the precipitate by centrifugation. The
precipitate was dissolved in 0.15 M-NaCl to give a final volume of one-third that of original serum. The solution was then dialysed against several changes of 0.15 M sodium chloride at 4°C for at least 24 hours. The diffusate was tested with barium chloride to assure the complete removal of the sulphate.

The antibody titre of the two antisera raised was tested using the Ouchterlony double diffusion technique (see section 2:11). Anti-(plasma membrane) antiserum was allowed to diffuse against normal rat serum; only antisera showing the best precipitation lines were pooled together, otherwise they were rejected. Anti-(liver cell cytosol) antiserum was tested against liver cytosol, and those showing precipitation lines on the Ouchterlony plate were pooled, otherwise discarded.

To prepare fluorescein-labelled antibodies, the globulin fraction was adjusted to a protein concentration of 20 mg/ml. 1 ml of 0.5 M bicarbonate/carbonate buffer, pH 9.0, and 3 ml of 0.15 M sodium chloride were added per 100 mg protein. Fluorescein iso-thiocyanate (FITC) (1 ml of 2.5 mg/ml) in bicarbonate/carbonate buffer was added dropwise to the vigorously stirred solution. The mixture was incubated for 2 hours at 30°C, then dialysed overnight against physiological phosphate buffered saline (PBS) at 4°C to remove the unbound dye. Finally, chromatography on Sephadex G-50 assured the removal of all the free dye from the mixture, and the first emerging fraction was collected. Due to the interference of the bound FITC with various methods of protein estimation, a complete recovery of protein was assumed. The antisera were further concentrated using a "Diaflo" with a PM-10 membrane, to give the required protein concentration used for various estimations.
A quantitative method was used to investigate the extent of cross-reaction between rat liver plasma membrane and rat serum. In this study fluorescein-labelled anti-(rat serum) antiserum, prepared as described in section 2:7, and isolated plasma membrane were used. Such measurements were performed by incubating the fluorescein-labelled anti-(rat serum) antiserum with the membrane resuspended in PBS, at 37° for 30 minutes. After incubation the membranes were pelleted by centrifugation at 40,000 revs/min for 90 minutes. Loosely bound materials were removed by resuspending the membrane in an appropriate volume of PBS and repelleting. Sodium deoxycholate was added to give a final concentration of 1% and the fluorescence was measured at 520 nm with excitation at 494 nm.

To determine the percentage of plasma membrane antigens removed by the salt extraction procedure (see section 2:6), isolated and salt-extracted plasma membranes were incubated with the fluorescein-labelled anti-(plasma membrane) antiserum and processed exactly as above.
Immobilised anti-(plasma membrane) and anti-(cytosol) antisera were prepared by the method of Cuatrecasas et al. (1968). Such procedure involves the interaction of the imidocarbonate derivative of Sepharose-4B (CNBr-activated Sepharose) with the antibody protein in moderately alkaline medium.

a) Preparation of activated Sepharose

The imidocarbonate derivative of Sepharose-4B was prepared by reaction with cyanogen bromide. Sepharose-4B was washed thoroughly with distilled water and then was resuspended in an equal volume of distilled water. An equal volume of a freshly prepared aqueous solution of cyanogen bromide (100 mg/ml) was added, and the pH was adjusted immediately to, and maintained at, 11 by titrating the mixture with 4 N NaOH with constant stirring. When the reaction ended (about 8-10 min), the Sepharose was washed with about 20 volumes of cold water on a Buchner funnel under suction (about 2 min) followed by 20 volumes of cold 0.1 M bicarbonate, pH 9.0. The washed activated Sepharose was resuspended in a volume of 0.1 M bicarbonate, pH 9.0, equal to that of the original Sepharose.

b) Preparation of immobilised antisera

Anti-(plasma membrane) or anti-(cytosol) antiserum was added to
the activated Sepharose suspension as quickly as possible. The mixture was left gently stirring at 4° for 24 hours. The slurry was poured into a 1.5 x 50 cm column, allowed to pack and washed extensively with 0.15 M-NaCl followed by thorough washing with solutions used for elution (see section 2:10 below). Exposure of the column to each elution solution was followed by thorough washing with 0.15 M-NaCl.
In order to establish the exact conditions for the affinity chromatography technique to be used to separate rat serum antigens common to liver plasma membranes, cytosol and anti-(cytosol) antiserum were taken as a model of specific antigen-antibody binding phenomenon.

a) **Verification of antigen separation conditions**

In this preliminary experiment, liver cell cytosol and Sepharose-anti-(cytosol) conjugate (prepared as above) were left with gently mixing at 4°C for 30 minutes, 1 hour, 2, 4, 12 and 24 hours. The suspension was then packed in a 1.5 x 50 cm column and washed with 0.15 M-NaCl until no protein was detected in the washing (judged by the 280 nm measurement). The following solutions (500 ml each) were used to elute the bound antigens from the antibodies as quickly as possible.

- 50 mM Glycine-HCl, pH 2.8
- 1 M Propionic acid, pH 2.5
- 3 M Sodium thiocyanate, 50 mM phosphate, pH 6
- 2.5 M Magnesium chloride

The eluents were either neutralised to pH 7.0 (in case of acidic solutions) and/or dialysed against several changes of 0.15 M-NaCl (in case of solutions interfering with either enzyme or protein assays). By such experiments, it was possible to judge the elution power of each solution as measured by recovery of protein and enzyme activity.
b) Isolation of rat serum antigens common to liver plasma membrane

The immobilised anti-(liver plasma membrane) fraction (prepared as above) resuspended in an equal volume of 0.15 M-NaCl was mixed with rat serum. In early experiments 3 ml of 35 mg/ml rat serum protein was added dropwise to the suspended Sepharose-anti-(plasma membrane) conjugate and was incubated with gentle stirring, at 4°C. In initial experiments varying incubation times were tried. It was concluded that the best results were obtained if the incubation was extended to 24 hours. After incubation, the slurry was poured into the column, allowed to pack and treated exactly as in part a.

2.5 M-Magnesium chloride was used routinely as an eluent (see results section), elution was continued until no further protein was present in the eluent (measured as absorbance at 280 nm). Fractions of the protein peak were pooled and dialysed against 3 x 2 of 0.15 M-NaCl in an overnight sequence. By such procedure the percentage of serum protein common to liver plasma membrane was measured. Later the quantity of rat serum was increased gradually until a 50 ml Sepharose-anti-(plasma membrane) column containing 3 mg anti-(plasma membrane) protein per ml Sepharose was saturated. Routinely 10 ml rat serum of 35 mg/ml protein concentration was used.
All polyacrylamide gel electrophoresis was performed in 9 cm x 5 mm glass tubes using a home-made electrophoresis apparatus. The following solutions were used:

Solution (A) 36.6 gm Tris dissolved in distilled water to which 0.46 ml TEMED (NNNN-tetramethylethylenediamine) was added, the pH brought to 8.0 with HCl and the volume made up to 100 ml.

Solution (B) 20 gm Acrylamide and 0.53 gm Bis-acrylamide in 100 ml distilled water.

Solution (C) Freshly prepared 0.14% ammonium persulfate.

The polyacrylamide gels were prepared from 2 ml of A, 4 ml B, 8 ml C and 2 ml distilled water. The tubes were filled with this solution up to 8 cm height and carefully overlaid with distilled water. After polymerization, (30 min) samples (to which a few sucrose crystals were added) were layered on top of the polyacrylamide. Electrophoresis was performed for 2 hours at room temperature in Tris (0.05 M)-glycine (0.4 M) buffer, pH 8.3, using 5 mA/tube.

For all immuno-electrophoresis techniques described in this thesis, 1% w/v agar in 0.044 M-barbitone buffer, pH 8.6, was used. The agar plates were prepared by pouring 10 ml of molten agar on an 8 x 8 cm glass plate giving 3 mm gel thickness. Wells and troughs were cut using a home-made cutter. The dimensions are shown in Fig 2-5a. After clearing the wells, a drop of the antigen solution was loaded and
electrophoresed at room temperature for 2 hours with the current adjusted to 20 mA/plate (about 10 volt/cm across the plate). The troughs were then removed and the antisera were loaded immediately.

Ouchterlony double diffusion technique was performed in 1.5% w/v agar in 0.3 M phosphate buffer, pH 8.6, on plates prepared as described above. The dimensions of the home-made Ouchterlony type cutter are shown in Fig. 2-5b.

Diffusion and precipitation were allowed to proceed for 2-3 days at room temperature before the plates were washed with several changes of 2.5 % NaCl followed by distilled water. The plates were then dried using hot air. Dry plates were then stained for protein with 2% Ponceau S in 2% acetic acid for 30 min and were then washed with 2% acetic acid until a clear background was obtained. In some cases the dry plates were stained for protein with Coomassie Brilliant blue R for 10 min and were then washed with 45% ethanol - 10% acetic acid.

Polyacrylamide gels were stained either for total carbohydrates by the periodic acid Schiff procedure (Zacharias et al., 1969) or for protein by incubating the gels in 2% Ponceau S overnight. The gels were then washed with 2% acetic acid until the areas containing no protein are completely destained.

For histochemical staining on the immuno-electrophoresed plates, 7 μl of the detergent extract (see section 2:6), containing about 25 mg/ml protein, were electrophoresed at 4° for 6 hours using 4 mA/plate (approx 40 volts). The troughs were then removed and the antisera were added immediately and allowed to diffuse for 24 hours.
at room temperature followed by two days at 4°. The unprecipitated proteins were washed out with 2.5 M-NaCl (as above) and the wet gels were stained as described below.

Fig 2-5

(a) Dimensions of the wells and troughs used for agar gel electrophoresis.
(b) Dimensions of the Ouchterlony type cutter used.
a) **Enzymes releasing inorganic phosphate**

Staining was performed according to the method of El-Aaser *et al.* (1973). The incubation mixture contained 50 mM dimethylglutarate buffer, pH 7.2, 2 mM lead nitrate, 5 mM magnesium nitrate and 5 mM AMP or ATP as substrate. After incubation for 2-3 hours at 37°C, the plates were washed extensively with distilled water and immersed in 2% yellow ammonium sulfide. After a few moments the plates were thoroughly washed with distilled water and dried. Although a precipitate (perhaps lead phosphate) does form in the incubation mixture, it seems not to interfere with the enzyme staining.

b) **Alkaline phosphatase**

The method of Gomori (1951) was used for alkaline phosphatase staining. The plates were incubated at 37°C with a mixture containing 10 mg sodium-α-naphthyl phosphate, 40 ml Tris-HCl buffer (0.25 M), pH 8.0; 40 ml distilled water; 0.25 ml 10% MgCl_2 and 30 mg diazonium salt "Blue RR". Three to four changes of the incubation mixture every 10-15 minutes was found to give the best staining. The plates were then washed with distilled water and dried.

c) **L-Leucyl-β-naphthyl amidase**

Application of the method of Gomori (1954) for the histochemical staining of the aminopeptidase was found to be the most suitable.

One ml of 1% aqueous stock solution of L-leucyl-β-naphthyl amide
was diluted forty times with water and mixed with 10 ml of 0.2 M Tris-HCl buffer, pH 7.1. 30 mg of diazotized o-aminazoletoluene (Garnet GBC salt) are added. The gels were incubated in this mixture at 37° until the desired staining reaction was obtained (usually about one hour). The incubation medium must be changed every 15-20 minutes. The gels were then washed with water and either dried (in case of agar gels) or stored in 2% acetic acid (in case of polyacrylamide gels).
CHAPTER THREE

RESULTS
3 : 1) PURIFICATION OF PLASMA MEMBRANE FROM NUCLEAR FRACTION

As discussed in the Introduction various authors have shown that, after homogenisation, the plasma membrane breaks into large sheets which sediment with the nuclear fraction and small vesicles recovered in the microsomal fraction. Purification of plasma membrane sheets from crude nuclear fraction has already been reported from this laboratory (Hinton et al., 1970) and this procedure was used in early experiments in the present study. The nuclear fraction, prepared from perfused liver, was centrifuged at 3700 revs/min for 50 min in an AXII zonal rotor. After centrifugation four distinctive bands of particles could be seen in the rotor (Fig. 3-1a, regions 2-5). The distribution of marker enzymes for various subcellular structures, namely 5'-nucleotidase, succinate dehydrogenase, glucose 6-phosphatase and acid phosphatase, indicated that the four regions correspond respectively to: very low sedimenting material including microsomes (2), mitochondria (3), plasma membrane (4) and nuclei contaminated with aggregated material (5). The lysosomes, as indicated by the distribution of acid phosphatase, were mainly located between the mitochondria and the microsomal bands (Fig. 3-2). These fractions have been examined by
Fig 3-1

Pattern obtained after centrifugation for 50 min at 3700 revs/min in an AXII zonal rotor of:

a) A crude nuclear fraction separated from a rat liver homogenate by differential pelleting.

b) Material floated from the whole homogenate after adjusting the density to 1.19 and centrifugation for 90 min at 40000 revs/min.

In both a and b perfused liver was used, and the sample, resuspended in 0.25 M sucrose, was layered in front of the gradient as indicated by the arrows. All solution contained 5mM-NaHCO₃, pH 7.4
Fig 3-2

Distribution of protein and marker enzymes after centrifugation of a crude nuclear fraction for 50 minutes at 3700 revs/min in an AXII zonal rotor.

Top: ——, density; ——, protein; ——, glucose 6-phosphatase (G6Pase); ——, 5'-nucleotidase (AMPase); bottom: ——, acid β-glycerophosphatase (AcidPase); ——, succinate dehydrogenase (SuccDase); ——, L-leucyl-β-naphthylamidase (LNase).
electron microscopy (Fitzsimons, 1969), light microscopy and enzymology (Hinton et al., 1970), and it was shown that region (4) contains sheets of plasma membrane. Therefore, in this study, enzymology was taken as criteria for the purity of this liver fraction. Examination of Fig. 3-1a shows the presence of a peak (region 1) with no enzyme activities. This peak is almost certainly composed of lipid material which has floated from the sample region.

As shown in Fig. 3-2, the plasma membrane sheets of region (4) still possess a considerable amount of glucose 6-phosphatase and succinate dehydrogenase. These activities have been attributed to the presence of mitochondria and endoplasmic reticulum trapped within the plasma membrane sheets (Hinton et al., 1970). The plasma membrane of region (4) was freed from most of the contaminating material by flotation from sucrose solution of density 1.19 g/ml. This flotation resulted in increasing the purification of 5'-nucleotidase from 14-fold to 30-fold as compared with the homogenate. Table 3-1 shows the purity and recovery of the plasma membrane fraction which was found to be almost a duplicate of that reported by Hinton et al. (1970).

Later in this study the procedure for isolating liver plasma membrane was modified by introducing a flotation step prior to the zonal centrifugation as it was described by Prospero and
Hinton (1973; "second procedure") in the separation of hepatoma plasma membrane (see Fig 2-4, Materials and Methods). This modification allowed for more material to be processed in a single centrifugation by reducing the amount of other subcellular fractions in the fraction loaded onto the A XII zonal rotor and so preventing overloading of the gradient. Fig. 3-1b shows, as expected, that centrifugation of the floated material from the whole homogenate in an A XII zonal rotor gives one major protein region which corresponds to region (4) of Fig. 3-1a already identified as the plasma membrane sheets. The plasma membrane marker enzymes, namely 5'-nucleotidase, L-leucyl-β-naphthylamidase, alkaline p-nitrophenyl phosphatase, alkaline phosphodiesterase and alkaline ribonuclease, were all concentrated in the same band (region 4) of the gradient (Fig. 3-3). The modification of the isolation procedure resulted in an increase in 5'-nucleotidase specific activity from 14-fold (method of Hinton et al., 1970) to 17-fold over that of the homogenate. Flotation of the plasma membrane sheets so prepared, from sucrose solution of density 1.19 resulted in further purification of 5'-nucleotidase specific activity to about 37-fold over the homogenate (Table 3-2).

In any cell fractionation technique the possibility exists that the isolated fraction could be contaminated by proteins absorbed after breakage of the cell membrane. In the case of liver cell plasma membrane Emmelot et al. (1964) reported that non-membraneous protein can be removed from the isolated plasma
Fig 3-3

Distribution of marker enzymes after centrifugation of the floated material from the whole homogenate for 50 minutes at 3700 revs/min in an AXII zonal rotor.

a) - - - - - , density; \( \circ \) - - - o, protein; \( \circ \) - - - - o, 5' nucleotidase (AMPase); \( \square \) - - - - \( \square \), glucose 6-phosphatase (G6Pase).

b) \( \times \) - - - \( \times \), L-leucyl -\( \beta \)-naphthylamidase (LNase); \( \times \) - - - \( \times \), succinate dehydrogenase (SuccDase); \( \circ \) - - - \( \circ \), acid \( \beta \)-glycerophosphatase (Acid Pase).

Other plasma membrane enzymes, namely alkaline \( \rho \)-nitrophenyl phosphatase, alkaline phosphodiesterase (measured with bis- \( \rho \)-nitrophenyl phosphate as substrate) were all coincident in distribution with 5'-nucleotidase.
membrane by washing with 0.15 M-NaCl. It has also been reported that proteins trapped inside membrane vesicles are released by washing with 0.2 M-NaHCO₃ buffer, pH 9.0 (Weihing et al., 1972). Therefore, to avoid the possibility that our plasma membrane preparation could be contaminated by non-membraneous proteins, the isolated plasma membranes were exposed to 0.15 M-NaCl followed by 0.2 M-NaHCO₃ buffer, pH 9.0. Washing the isolated membranes with such solutions resulted in removal of a considerable amount of protein together with a significant proportion of plasma membrane marker enzymes. Table 3-3 shows that the proportion of proteins and plasma membrane marker enzymes extracted by the bicarbonate buffer is much higher than that removed by 0.15 M-NaCl. These results may be explained in two ways:— 1) the materials contaminating our plasma membrane preparation are associated with such enzymic activities and that the isolated plasma membranes are contaminated with a large number of vesicles; 2) that the washing procedure does in fact remove membrane proteins to some extent. Material removed by the bicarbonate buffer and 0.15 M-NaCl, was further examined by testing it against anti-(liver cell cytosol) and anti-(liver cell plasma membrane) antisera prepared as described in Chapter 2 "Materials and Methods".
Table 3-1: Purification and yield of various marker enzymes during preparation of plasma membrane fraction by centrifugation of a crude nuclear fraction in an A XII zonal rotor for 50 min at 3,700 revs/min.

The results are given as means ± standard deviation, with number of plasma membrane preparation examined (in parentheses).

**5'-Nucleotidase**

<table>
<thead>
<tr>
<th>Specific Activity</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>umoles/mg Protein/hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>2.9 ± 0.54 (10)</td>
<td>100</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>7.25 ± 0.83 (10)</td>
<td>39.9 ± 2.7</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1a)</td>
<td>42.34 ± 3.8 (10)</td>
<td>8.74 ± 0.8</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>87.58 ± 2.45 (10)</td>
<td>6.92 ± 1.1</td>
</tr>
</tbody>
</table>

**Glucose 6-Phosphatase**

<table>
<thead>
<tr>
<th>Specific Activity</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>umoles/mg Protein/hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>2.34 ± 0.61 (10)</td>
<td>100</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>3.53 ± 1.22 (10)</td>
<td>13.21 ± 1.6</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1a)</td>
<td>1.38 ± 0.46 (10)</td>
<td>0.23 ± 0.12</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>0.88 ± 0.4 (10)</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>
Table 3-1 Continued.

**Acid Phosphatase**

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles/mg Protein/hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>2.21 ± 0.41 (10)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>1.72 ± 0.39 (10)</td>
<td>11.32 ± 1.81</td>
<td>0.78</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1a)</td>
<td>0.66 ± 0.79 (10)</td>
<td>0.13 ± 0.1</td>
<td>0.299</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>0.71 ± 0.162 (10)</td>
<td>0.06 ± 0.05</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Succinate dehydrogenase**

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles/mg Protein/hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>1.61 ± 0.12 (10)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>1.932 ± 0.83 (10)</td>
<td>18.92 ± 2.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1a)</td>
<td>0.56 ± 0.36 (10)</td>
<td>0.22 ± 0.27</td>
<td>0.35</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>0.145 ± 0.02 (10)</td>
<td>0.019 ± 0.01</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Table 3-1 Continued.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>I-leucyl-β-naphthylamidase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity ugm/mg Protein/hr.</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>89.4 ± 2.39 (10)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>189.5 ± 1.18 (10)</td>
<td>29 ± 1.5</td>
<td>2.12</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1a)</td>
<td>1349.9 ± 6.4 (10)</td>
<td>7.81 ± 0.72</td>
<td>15.1</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>2869 ± 9.42 (10)</td>
<td>7.2 ± 0.37</td>
<td>32.1</td>
</tr>
</tbody>
</table>

| **Alkaline Phosphatase** |

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity ugm/mg Protein/hr.</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>32.8 ± 4.5 (10)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>75.76 ± 3.77 (10)</td>
<td>29.5 ± 1.7</td>
<td>2.31</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1a)</td>
<td>524.8 ± 15.6 (10)</td>
<td>8.1 ± 0.42</td>
<td>16</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>724.88 ± 9.8 (10)</td>
<td>5.2 ± 0.91</td>
<td>22.1</td>
</tr>
</tbody>
</table>
### Table 3-1 Continued

#### Catalase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>5.695 (1)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Nuclear fraction</td>
<td>2.1 (1)</td>
<td>5.75</td>
<td>0.36</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1a)</td>
<td>Undetectable</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>Undetectable</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Unit activity of the enzyme is given as an arbitrary unit equivalent to the amount of enzyme which gives log OD of 1 under the assay conditions as given by Leighton et al. (1968).
Table 3-2: Purification and yield of various marker enzymes during preparation of plasma membrane by centrifugation of the floated material from the whole liver homogenate for 50 min at 3700 revs./min in an A XII zonal rotor.

The results are given as means ± standard deviation, with the number of plasma membrane preparation examined (in parentheses).

<table>
<thead>
<tr>
<th>5'-Nucleotidase</th>
<th>Specific Activity</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2.85 ± 0.61 (14)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>14.82 ± 0.74 (14)</td>
<td>40.1 ± 3.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1b)</td>
<td>49.02 ± 1.08 (14)</td>
<td>9.3 ± 2.1</td>
<td>17.2</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>106.87 ± 1.75 (14)</td>
<td>8.9 ± 1.6</td>
<td>37.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose 6-phosphatase</th>
<th>Specific Activity</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2.65 ± 0.94 (14)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>1.93 ± 0.35 (14)</td>
<td>11.8 ± 0.42</td>
<td>0.73</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1b)</td>
<td>1.08 ± 0.085 (14)</td>
<td>0.46 ± 0.53</td>
<td>0.41</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>0.016 ± 0.013 (14)</td>
<td>0.05 ± 0.012</td>
<td>0.006</td>
</tr>
</tbody>
</table>
### Table 3-2 Continued

<table>
<thead>
<tr>
<th>Method</th>
<th>Acid Phosphatase</th>
<th>Succinate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Umoles/mg Protein/hr.</td>
<td>% Recovery</td>
</tr>
<tr>
<td>Homogenate</td>
<td>2.13 ± 0.78 (14)</td>
<td>100</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>0.19 ± 0.088 (14)</td>
<td>7.43 ± 0.96</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1b)</td>
<td>0.019 ± 0.014 (14)</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>0.014 ± 0.006 (14)</td>
<td>0.05 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>Specific Activity</td>
<td>% Recovery</td>
</tr>
<tr>
<td>Homogenate</td>
<td>1.51 ± 0.21 (14)</td>
<td>100</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>0.46 ± 0.1 (14)</td>
<td>8.6 ± 0.93</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1b)</td>
<td>0.021 ± 0.09 (14)</td>
<td>0.091 ± 0.011</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>Undetectable</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3-2 Continued

<table>
<thead>
<tr>
<th>L-leucyl-β-naphthylamidase</th>
<th>Specific Activity ug/mg Protein/hr.</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>91.81 ± 2.27 (8)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>206.57 ± 3.1 (8)</td>
<td>30 ± 2.2</td>
<td>2.25</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1b)</td>
<td>1670.9 ± 8.42 (8)</td>
<td>9.2 ± 1.13</td>
<td>18.2</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>3598.95 ± 9.52 (8)</td>
<td>8.99 ± 2.1</td>
<td>39.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Alkaline Phosphatase</th>
<th>Specific Activity ug/mg Protein/hr.</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>14.28 ± 2.6 (5)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>44.26 ± 7.3 (5)</td>
<td>30.21 ± 2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1b)</td>
<td>249.9 ± 10.1 (5)</td>
<td>8.7 ± 1.4</td>
<td>17.5</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>458.3 ± 8.8 (5)</td>
<td>8.5 ± 0.6</td>
<td>32.1</td>
</tr>
</tbody>
</table>
Table 3-2 Continued

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ug/mg Protein/hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>32.2 ± 3.9 (5)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>86.94 ± 1.4 (5)</td>
<td>31.1 ± 1.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Zonal plasma membrane (central fraction of region 4, Fig.3-1b)</td>
<td>582.8 ± 1.52 (5)</td>
<td>9.3 ± 0.71</td>
<td>18.1</td>
</tr>
<tr>
<td>Purified plasma membrane</td>
<td>1246.14 ± 8.6 (5)</td>
<td>9.1 ± 1.1</td>
<td>38.7</td>
</tr>
</tbody>
</table>
Table 3-3: Percentage of material removed by washing the isolated plasma membrane successively with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0.

The results are given as means ± standard deviation followed by numbers of observation (in parentheses)

<table>
<thead>
<tr>
<th>Protein</th>
<th>0.15 M-NaCl wash</th>
<th>0.2 M-bicarbonate wash</th>
<th>Washed membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>6.62 ± 1.1</td>
<td>27.5 ± 2.6</td>
<td>67.1 ± 1.4</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>2.1 ± 0.71</td>
<td>7.1 ± 0.9</td>
<td>82.8 ± 1.6</td>
</tr>
<tr>
<td>L-leucyl-β-naphthylamidase</td>
<td>1.67 ± 0.43</td>
<td>2.23 ± 0.75</td>
<td>93.3 ± 2.6</td>
</tr>
<tr>
<td>Ribonuclease assayed at pH 7.8</td>
<td>3.2, 2.6</td>
<td>4.1, 6.2</td>
<td>86.1, 87.2</td>
</tr>
</tbody>
</table>

The results are given as percentages of the protein or enzyme activity found in membranes stored in 0.15 M-NaCl at 4°C during the 48 hours required for the washing procedure.

Each washing step was carried out by incubating the membrane with the washing medium overnight at 4°C, and the washed membrane was recovered by spinning for 90 min at 40,000 revs/min.
3 : 2) PROPERTIES OF ANTISERA PREPARED

a) Anti-(liver cell cytosol) antiserum

Rabbit anti-(liver cell cytosol) was raised as described in Chapter 2 (Materials and Methods). The specificity of the anti-(cell cytosol) so prepared was tested against liver cell cytosol prepared from perfused liver by immunoelectrophoresis (Fig. 3-4).

Fig. 3-4. Immunoelectrophoresis of liver cytosol against anti-(cell cytosol). The antisera in troughs are from two different rabbits. The upper and lower wells contain about 0.25 mg cytosol protein, the central well contains the same cytosol diluted five times. Electrophoresis was for 2 hours at about 10 V/cm in 0.044 M-barbitone buffer, pH 8.6. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plate was stained for protein with Ponceau S.
b) **Anti-(liver cell plasma membrane) antiserum**

Anti-(liver cell plasma membrane) was prepared in rabbits against plasma membrane subjected to both 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0 (core plasma membrane). The specificity of the anti-(plasma membrane) was tested against isolated plasma membranes and that recovered after washing with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0. The anti-(plasma membrane) was tested as described below. Briefly, fluorescein-labelled anti-(plasma membrane) antiserum was allowed to react directly with the membrane and the fluorescence attached to the membrane was measured. The presence of antibodies against plasma membrane enzymes was verified by histochemical staining methods using solubilized plasma membrane.

(i) **Binding of anti-(plasma membrane) to isolated plasma membrane**

To measure the extent of binding of the prepared anti-(plasma membrane) antiserum to the isolated plasma membrane, aliquots of the isolated membrane containing 0.1 mg protein were incubated at 37° for 30 min with an increasing amount of fluorescein-labelled anti-(plasma membrane). The membrane was then washed with phosphate buffered saline (PBS) to remove the loosely bound material and the fluorescence intensity of the antigen-antibody complex was measured (as described in Chapter 2, Materials and Methods). Initially the extent of binding of labelled anti-(plasma membrane) to the membrane proteins was found to increase linearly
as the amount of labelled anti-(plasma membrane) incubated in the assay medium was increased. However, after addition of 25 mg anti-(plasma membrane) globulin to the incubation mixture, the intensity of the fluorescence measured at 520 nm with excitation at 494 nm was found to be almost constant (Fig. 3-5a). This suggested that the antigenic binding sites of the isolated plasma membrane were saturated. Saturation of plasma membrane binding sites was achieved when about 30 mg of anti-(plasma membrane)globulin were bound per mg plasma membrane proteins. Calculation from a standard curve of fluorescein labelled anti-(plasma membrane)(Fig. 3-5b) showed that about 12% of the proteins in the anti-(plasma membrane) globulin preparation were capable of binding to the membrane.

It was shown earlier in this section that washing the isolated plasma membrane successively with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0 removed a considerable amount of protein from the membrane. This washing procedure apparently increased the binding capacity of the membrane to anti-(plasma membrane). The fluorescence intensity measured after incubating the washed plasma membrane with sufficient anti-(plasma membrane), enough to saturate the isolated plasma membrane, was enhanced by about 10%. These results indicated that washing the isolated plasma membrane with 0.15 M-NaCl and 0.2 M-bicarbonate resulted in the exposure of new antigenic binding determinants.
a) Binding of fluorescein-labelled anti-(plasma membrane) immunoglobulin to plasma membranes as a function of the amount of globulin added. In each case an aliquot containing 0.1 mg plasma membrane protein was incubated with the immunoglobulin at 37°C for 30 min. Sodium deoxycholate was added to each assay to a final concentration of 1%.

b) Fluorescence intensity as a function of anti-(plasma membrane) globulin concentration.

Fig 3-5
which were previously masked. The masking proteins could be either materials absorbed on to the plasma membrane during the isolation procedure, or they are true membrane proteins which hide other potential binding sites located deeper in the membrane. These two possibilities were further examined as will be described later.
(ii) Antibodies against plasma membrane enzymes

The presence of antibodies against plasma membrane enzymes in our anti-(plasma membrane) globulin fraction preparation was examined by histochemical staining of the immunoprecipitation lines formed by solubilized plasma membrane against the anti-(plasma membrane) globulin upon electrophoresis. Since all the results presented in this section were obtained after solubilizing the plasma membrane by 1% sodium deoxycholate + 0.5% Lubrol W, the effect of this mixture of detergents on the activity of plasma membrane enzymes was examined. The results of these studies (Fig.3-6) show that the mixture of detergents used has no effect on the assay of the membrane protein by the Lowry method. However, 5'-nucleotidase was found to be activated by a factor of three, whereas L-leucyl-β-naphthylamidase and alkaline phosphatase were inhibited by about 30% and 45% respectively. These factors were used in the calculation of the amount of material solubilized after treatment of the membrane with the detergent. The results presented in Table 3-4 show that about half of the plasma membrane proteins are solubilized by the detergent mixture. The enzyme activities were mainly associated with the solubilized part of the plasma membrane. The failure to obtain a 100% recovery could be explained as either due to the
Effect of sodium deoxycholate (DOC) + Lubrol W on protein, assayed by Lowry method, and enzyme activities of rat liver plasma membrane. In all cases, an aliquot containing 0.2 mg plasma membrane protein was mixed with the corresponding amount of concentrated stock solution of the detergent mixture to give a final concentration of 1% DOC + 0.5% Lubrol W. The values on the X-axis show only the DOC concentration of the detergent mixture.

a) Contribution of the detergents used for solubilization to the protein assayed by the Lowry method. Reagent blank assayed by substituting 0.15 M-NaCl for the amount of tissue used. In case of enzyme assays the "Reagent blank" was negligible.

b) Activity of plasma membrane enzymes and protein in the presence of the detergent mixture as a percentage of control assayed by substituting 0.15 M-NaCl for detergent mixture.
5'-Nucleotidase (AMPase).
L-leucyl-β-naphthylamidase (LNase).
Alkaline phosphatase (APNPPase).
Inaccuracy of the correction factors, or the residue recovered after centrifugation trapping some of the detergent.

Table 3-4: Percentage of plasma membrane protein and enzyme activities extracted with 1% sodium deoxycholate + 0.5% Lubrol W. The results are given as means ± standard deviation, with number of observations in parentheses.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Extract</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>55 ± 2.5 (3)</td>
<td>42.9 ± 1.6 (3)</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>90 ± 3.7 (5)</td>
<td>35.6 ± 1.2 (5)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>56.2 ± 2.3 (3)</td>
<td>11.9 ± 1.4 (3)</td>
</tr>
<tr>
<td>L-leucyl-β-naphthylamidase</td>
<td>60.7 ± 1.9 (5)</td>
<td>19.3 ± 3.6 (5)</td>
</tr>
</tbody>
</table>

In all cases plasma membrane preparation washed with 0.15 M-NaCl and 0.2 M-NaHCO₃ containing about 12 mg protein was resuspended in 10 ml of 1% sodium deoxycholate + 0.5% Lubrol W. Solubilization was carried out for 24 hours at 4°C. The residue collected by centrifugation at 40,000 revs/min for 90 min was resuspended in 0.15 M-NaCl. The results are given as a percentage of the protein or enzyme activity found in membranes stored in 0.15 M-NaCl at 4°C during the 24 hours required for the solubilization procedure after correcting for the effect of the detergents on the measurement as presented in Fig. 3–6.
Immunoelectrophoresis of the solubilized plasma membrane against anti-(plasma membrane) globulin was carried out as described in "Materials and Methods". At least nine precipitation lines were detected when the plate was stained for protein (Fig. 3-7a). The presence of antibodies against plasma membrane enzymes was demonstrated by histochemical staining of the wet gel. 5'-Nucleotidase activity was found in two closely spaced precipitation lines (Fig. 3-7b). ATPase activity was found in six precipitation lines (Fig. 3-7c); three of these lines were found to be associated with both L-leucyl-β-naphthylamidase and alkaline phosphatase activities (Fig. 3-7d,e).
Fig. 3.7

Immunoelectrophoresis plates of solubilized plasma membrane against anti-(plasma membrane) globulin. In all cases about 0.2 mg of the solubilized plasma membrane protein was electrophoresed at 4° for 6 hours at about 4 mA/plate in 0.044 M-barbitone buffer, pH 8.6. The unprecipitated proteins were washed off with 2.5% NaCl and the plates were:

a) Stained dry, for protein with Coomassie Brilliant blue R.
   Nine precipitation lines are detected.

b) Stained wet, for 5′-nucleotidase activity with AMP as substrate by the method of El-Aasser et al. (1973). Two active lines with the same mobility were visible immediately after staining.

c) Stained wet, for nucleoside triphosphatase by the method of El-Aasser et al. (1973) with ATP as substrate. Six active precipitates are seen.

d) Stained wet, for L-leucyl-β-naphthylamidase by the method of Gomori (1954) with L-leucyl-β-naphthylamide as substrate. Three enzymatically active precipitation lines are seen.

e) Stained wet, for alkaline phosphatase by the method of Gomori (1951) with sodium-α-naphthyl phosphate as substrate. Three active precipitates are seen. The faint line which appears at the edge of the slot is due to the thick precipitation line shown in (a) and is not an alkaline phosphatase active line.
The material extracted from our plasma membrane preparation during the washing procedure was characterized immunologically by Ouchterlony double diffusion and by Immunelectrophoresis. Both the 0.15 M-NaCl and the 0.2 M-NaHCO$_3$ extracts were concentrated up to about 25 mg/ml protein and an aliquot of the concentrate was allowed to diffuse against anti-(rat serum), anti-(cell cytosol) and anti-(plasma membrane) antisera. This experiment was used to elucidate two points, first whether the 0.15 M-NaCl and 0.2 M-NaHCO$_3$ washes removed identical proteins from the isolated plasma membrane, second whether the three antisera contain antibodies to the same components of the two washes.

The results presented in Fig. 3-8 show that both washes contain materials that are capable of reacting with all of the three antisera. Observations deduced from the pattern formed by both washes against all three antisera were summarized in Table 3-5. For convenience, the lines formed by both washes against anti-(rat serum), anti-(cell cytosol) and anti-(plasma membrane) are designated as N, C and P lines with a subscript which indicates
Ouchterlony double diffusion plates of the concentrated material extracted by 0.15 M-NaCl and 0.2 M-NaHCO$_3$ against

a) Anti-(rat serum) antiserum (A/S)
b) Anti-(liver cell cytosol) antiserum (A/C)
c) Anti-(liver plasma membrane) antiserum (A/PM).

In the outside wells an aliquot containing about 14 mg protein was placed of:

1) 0.15 M-NaCl extract
2) 0.2 M-NaHCO$_3$ extract
3) liver cell cytosol
4) rat serum.

In all cases diffusion and precipitation was allowed to proceed for 48 hours at room temperature. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plates were stained for proteins with Ponceau S.
Table 3-5: Observations deduced from Ouchterlony double diffusion plates of 0.15 M-NaCl and 0.2 M-NaHCO₃ washes against anti-(rat serum), anti-(cell cytosol) and anti-(plasma membrane) antisera, shown in Fig. 3-8.

The precipitation lines were numbered in a way similar to that described in Fig. 3-8.

<table>
<thead>
<tr>
<th>Diffusion against</th>
<th>Precipitation lines formed by</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.15 M-NaCl (SW)</td>
<td>0.2 M-NaHCO₃ (BW)</td>
</tr>
<tr>
<td>Anti-(rat serum) antiserum</td>
<td>N₁, , N₃, N₄</td>
<td>N₁, N₂, N₃, N₄</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N₂ is missing from SW</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N₃ is more pronounced in BW than in SW</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N₄ is more pronounced in SW than in BW</td>
</tr>
<tr>
<td>Anti-(cell cytosol) antiserum</td>
<td>C₁, C₂, C₃</td>
<td>C₁, C₂, C₃</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C₂ is more pronounced in SW than in BW</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C₃ is much more pronounced in BW than in SW</td>
</tr>
<tr>
<td>Anti-(plasma membrane) antiserum</td>
<td>P₁, P₂</td>
<td>P₁, P₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both are relatively of the same strength.</td>
</tr>
</tbody>
</table>
the position of the line relative to the antigen well. A problem
in interpreting these results is caused by the formation of
precipitation lines by cell cytosol against anti-(rat serum) antiserum
(Fig. 3-8a). This would indicate that our cytosol preparation
contains material that is homologous to rat serum proteins, hence
one would expect that our anti-(cytosol) preparation contains
antibodies against such proteins. This brings the possibility that
the precipitation lines \((C_1, C_2, \text{ and } C_3)\) formed by both washes
against anti-(cell cytosol) (Fig. 3-8b) do not necessarily represent
cytosol proteins, but are due to the presence of serum proteins.

For convenient presentation of the results shown in Fig. 3-9
the precipitation lines formed against all three antisera by 0.15 M-
NaCl wash were designated as the "S" lines and those formed by
0.2 M-NaHCO\(_3\) wash as the "B" lines with a subscript which
indicates the position of the precipitation line relative to the
antigen well. Briefly Fig. 3-9a shows that the material extracted
by 0.15 M-NaCl forms three lines against anti-(rat serum), three
lines against anti-(cell cytosol), and two lines against anti-(plasma membrane) antisera. A slightly different pattern was
obtained from diffusion of 0.2 M-NaHCO\(_3\) wash against all three
antisera (Fig. 3-9b). Four lines were formed against anti-(rat
serum), five lines against anti-(cell cytosol) and two lines against
anti-(plasma membrane) antisera. Comparison of the precipitation
lines formed by both washes against the three antisera, shown
in Fig. 3-9, is presented in Table 3-6.
Ouchterlony double diffusion plates of the material extracted by 0.15M-NaCl (SW) and 0.2M-NaHCO₃ buffer (BW) against anti-(liver plasma membrane) (4), anti-(cell cytosol) (2) and anti-(rat serum) (1) antisera.
Wells 3, 6 and 5 contain 1:5 dilution of (4), (2) and (1) respectively. Diffusion and precipitation was allowed to proceed for 48 hours at room temperature and the unprecipitated proteins were washed off with 2.5% NaCl. The dry plates were stained for proteins with Ponceau S. Both of the 0.15 M-NaCl and 0.2 M-NaHCO₃ extracts were concentrated so that an aliquot containing about 14 mg protein could be placed in the wells.
Table 3-6: Observations deduced from Ouchterlony double diffusion plates shown in Fig. 3-9 of 0.15 M-NaCl and 0.2 M-NaHCO₃ washes against anti-(rat serum), anti-(cell cytosol) and anti-(plasma membrane) antisera.

The precipitation lines were numbered in a way similar to that described in Fig. 3-9.

<table>
<thead>
<tr>
<th>Line number formed by 0.15 M-NaCl</th>
<th>The line common to</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁</td>
<td>B₁</td>
<td>Anti-(rat serum), anti-(cytosol) and anti-(plasma membrane)</td>
</tr>
<tr>
<td></td>
<td>B₂</td>
<td>Anti-(rat serum) and anti-(cytosol)</td>
</tr>
<tr>
<td>S₃</td>
<td>B₃</td>
<td>Anti-(rat serum) and anti-(cytosol)</td>
</tr>
<tr>
<td>S₄</td>
<td>B₄</td>
<td>Anti-(rat serum) and anti-(cytosol)</td>
</tr>
<tr>
<td>S₅</td>
<td>B₅</td>
<td>Anti-(cytosol) only</td>
</tr>
<tr>
<td>S₆</td>
<td>B₆</td>
<td>Anti-(plasma membrane) only</td>
</tr>
</tbody>
</table>
A problem in comparing the results presented in Table 3-5 with that in Table 3-6 is caused by the presence of five precipitation lines formed by 0.2 M-NaHCO$_3$ wash against anti-(cell cytosol) (Fig. 3-9b), while only three were detected in Fig. 3-8b. However, by comparing the position of the line relative to the antigen well and the cross reaction between the different antisera it is possible to say that precipitation line:

- $N_1$ is equivalent to $S_1$, $B_1$, $C_1$ and $P_1$.
- $N_2$ is equivalent to $B_2$ which is missing in the pattern of 0.15 M-NaCl.
- $N_3$ is equivalent to $S_3$, $B_3$ and $C_3$.
- $N_4$ is equivalent to $S_4$ and $B_4$.
- $C_2$ is equivalent to $S_5$ and $B_5$.
- $P_2$ is equivalent to $S_6$ and $B_6$.

This would indicate that at least six components were extracted from our plasma membrane preparation by 0.15 M-NaCl and 0.2 M-NaHCO$_3$ washes. One component, found in both extracts at the same concentration (line 1 above), reacts with all three antisera. Three components, present in both extracts but at different concentrations (lines 2, 3 and 4) are common to rat serum and cell cytosol, besides two components which are genuine cytosol and plasma membrane proteins respectively. The cytosol protein appears to be more concentrated in the 0.15 M-NaCl extract, whereas the genuine plasma membrane protein present in both extracts at the same concentration.
The material extracted by 0.15 M-NaCl and 0.2 M-NaHCO$_3$ washes were further examined by immunoelectrophoresis against the three antisera. The pattern formed by both extracts against anti-(rat serum) and anti-(cell cytosol) antisera (Fig. 3-10) shows two precipitation lines corresponding to proteins migrating at about the position of albumin and $\beta$-globulin. Fig. 3-11 shows that at least six precipitation lines could be detected on immunoelectrophoresis of either extract against anti-(plasma membrane) antiserum. Three are negatively charged proteins, one is found in the $\gamma$-globulin region beside two remaining at the origin. Examination of Fig. 3-11 shows that the two lines formed by the extracts against anti-(rat serum) are continuous with two of the lines that were formed against anti-(plasma membrane) antiserum.

The results presented above showed that the material from the 0.15 M-NaCl wash reacted more strongly with anti-(cell cytosol) than with anti-(rat serum) antiserum. The material extracted by 0.2 M-NaHCO$_3$ buffer reacted more strongly with anti-(rat serum) than with anti-(cell cytosol). The extent of reactivity against anti-(plasma membrane) was found to be almost equal.

By the methods described in this section it was possible to examine the material extracted by both washes from the isolated plasma membranes. It is difficult to examine the insoluble part of the membrane by application of the same techniques. Therefore an alternative quantitative method was developed to examine the antigens that are common to liver cell plasma membrane and rat serum as will be described in the following section.
Immunoelectrophoresis plate of the material extracted by 0.15 M-NaCl (SW) and 0.2 M-NaHCO₃ (BW) against anti-(rat serum) (A/S) and anti-(liver cell cytosol) (A/C) antisera. In all cases 7µl of the extract containing about 0.3 mg protein was electrophoresed for 2 hours at about 10 V/cm across the plate in 0.044 M-barbitone buffer, pH 8.6 at room temperature. The antiserum was added immediately and allowed to diffuse for 48 hours at room temperature. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plate was stained for protein with Ponceau S.
Immunoelectrophoresis plate, stained for proteins with Ponceau S, of the material removed from the isolated plasma membrane by washing with 0.15 M-NaCl (SW) and 0.2 M-NaHCO₃ (BW) against anti-(plasma membrane) (A/PM) and anti-(rat serum)(A/S) antisera. For immunoelectrophoresis conditions see Fig 3-10.
The presence of antigens common to rat serum and liver plasma membrane was examined using fluorescein-labelled anti-(rat serum) and isolated rat liver plasma membranes. In the early part of this investigation, rabbit anti-(rat serum) was purchased from Wellcome Reagents Ltd. The globulin fraction, separated from the whole serum, was labelled with fluorescein isothiocyanate (FITC) as was described in Chapter 2. In an initial trial experiment liver plasma membranes, isolated according to the method of Hinton et al. (1970), were fixed to a microscopic slide with acetone and allowed to react with the fluorescein-labelled anti-(rat serum) globulin at 37° for 30 minutes and then washed with physiological phosphate buffered saline (PBS). Examination of the membranes in a fluorescence microscope showed that a small amount of the fluorescein-labelled antibodies had bound to the membranes. This indicated that anti-(rat serum) globulin contained antibodies against proteins which are exposed on the surface of the isolated plasma membrane.

The degree of reactivity of the isolated plasma membrane and anti-(rat serum) globulin was then examined quantitatively by incubating the isolated plasma membrane with the fluorescein-
labelled anti-(rat serum) at 37° for 30 minutes. Loosely bound material was removed by washing the membrane with PBS, as described in Chapter 2, and the fluorescence intensity of the pellet, resuspended in PBS, was measured at 520 nm with excitation at 494 nm. When increasing amounts of the isolated membrane were incubated with a fixed amount of fluorescein-labelled anti-(rat serum), the fluorescence intensity of the plasma membrane pellet increased as the amount of the membrane protein was increased (Fig. 3-12a). During these experiments it was noticed that the suspension of the plasma membrane pellet possessed some turbidity which might render the fluorescence intensity measurement relatively inaccurate. Addition of sodium deoxycholate (DOC) to a final concentration of 1% in the assay medium produced almost a clear solution and about four-fold increase in the fluorescence intensity of the membrane suspension (Fig. 3-12a), therefore it was used routinely in all further experiments.

To account for any endogenous fluorescence, tissue blank was introduced by substituting PBS for the labelled anti-(rat serum). At the excitation wavelength used, no fluorescence was detected in the tissue blanks so prepared.

The results presented above indicate that the amount of anti-(rat serum) proteins bound to the membrane is linearly proportional to the amount of plasma membrane present in the assay medium. In further experiments the effect of changing the
Fig. 3.12

(a) Amount of fluorescein-labelled anti-(rat serum) immunoglobulin (Wellcome Reagents) bound as a function of the amount of plasma membrane protein.
In each case 4.6 mg of the immunoglobulin was incubated with the membrane protein for 30 minutes at 37°C. After removing the loosely bound material, the membranes were resuspended in 3 ml PBS and the fluorescence measured at 520 nm with excitation at 494 nm, and after the addition of DOC to a final concentration of 1% in the assay medium.

(b) Binding of fluorescein-labelled anti-(rat serum) immunoglobulin (Wellcome Reagents) to plasma membrane as a function of the amount of immunoglobulin added.
In all cases an aliquot containing 0.7 mg of the plasma membrane protein was incubated with the fluorescein-labelled anti-(rat serum) immunoglobulin for 30 min at 37°C. The loosely bound material was washed off with PBS and the membranes were resuspended in 3 ml PBS. The fluorescence intensity at 520 nm with excitation at 494 nm was measured after the addition of DOC to a final concentration of 1% in the assay medium.

(c) Fluorescence intensity of the immunoglobulin, prepared from the anti-(rat serum) purchased from Wellcome Reagents, as a function of the protein concentration.
A: Anti-(rat serum) globulin

Mg plasma membrane protein

B: Anti-(rat serum) globulin

Mg Anti-(rat serum) globulin

C: Anti-(rat serum) globulin

Mg Anti-(rat serum) globulin
amount of anti-(rat serum) was examined by incubating a fixed amount of plasma membrane proteins (0.7 mg) with an increasing amount of fluorescein-labelled anti-(rat serum). It was found that the fluorescence intensity measured increased linearly until it reached an approximately constant value, presumably corresponding to saturation of all available binding sites of the membrane. The plasma membranes were found to saturate when about 25 mg of the fluorescein-labelled anti-(rat serum) globulin was incubated with 0.7 mg plasma membrane proteins (Fig. 3-12b). The fluorescence intensity of the membranes treated with labelled anti-(rat serum) at the saturation point was compared with that of standard solutions of fluorescein-labelled anti-(rat serum) (Fig. 3-12c). It was found that only about 0.15 mg of anti-(rat serum) globulins were bound per mg protein of the isolated plasma membrane. Under the same conditions, only 0.04 mg of non-immune globulins were bound per mg membrane protein. These results indicate that about 0.4% of the protein in the globulin fraction was capable of binding to the membranes.

Later, during this investigation, rabbit anti-(rat serum) was purchased as globulin fraction from Mercia Ltd. (U.K.) to cut down the time spent in the separation of the globulin fraction from the whole serum as obtained from Wellcome Reagents. When some of the experiments described above were repeated, more antibodies were found to be capable of binding to the membrane than with the
Wellcome antiserum. Therefore the whole series of experiments were repeated exactly as above. The results presented in Fig. 3-13a show that the extent of binding of the fluorescein-labelled anti-(rat serum) with the isolated plasma membrane is enhanced by about 35% (compare with Fig. 3-12a). However, the amount of anti-(rat serum) proteins bound to the membrane is still linearly proportional to the amount of the membrane protein present in the assay medium. The effect of changing the amount of anti-(rat serum) was also studied, and as was expected the fluorescence intensity measured increased linearly until it is approximately constant (Fig. 3-13b). Saturation of all available binding sites of the plasma membrane was achieved when about 17 mg of the labelled antiserum globulin was incubated with 0.7 mg membrane protein. Measurement of the fluorescence intensity of standard solutions of the fluorescein-labelled antiserum globulin (Fig. 3-13c) showed that about 0.17 mg of the antiserum globulins were bound per mg plasma membrane. This indicates that about 1% of the antiserum globulin was capable of binding to the isolated membrane.

The results presented above demonstrate that the isolated plasma membrane is showing different binding capacity towards the two antisera used. This could be attributed to the possibility that the two antisera used are showing different antibody reactivity. To examine the possibility that the two anti-(rat serum) globulin
Fig. 3.13

(a) Amount of fluorescein-labelled anti-(rat serum) immunoglobulin (Mercia Ltd.) bound as a function of the amount of plasma membrane protein. In all cases 4.6 mg of the immunoglobulin was incubated with the membrane protein for 30 minutes at 37°C. After removing the loosely bound material, the membranes were resuspended in 3 ml PBS and the fluorescence measured at 520 nm with excitation at 494 nm in the presence of 1% final concentration of DOC in the assay medium.

(b) Binding of fluorescein-labelled anti-(rat serum) immunoglobulin (Mercia Ltd.) to plasma membrane as a function of the amount of immunoglobulin added. In all cases an aliquot containing 0.7 mg of the membrane protein was incubated with the fluorescein-labelled anti-(rat serum) immunoglobulin for 30 minutes at 37°C. The loosely bound material was washed off with PBS and the membranes were resuspended in 3 ml PBS. The fluorescence intensity at 520 nm with excitation at 494 nm was measured after the addition of DOC to a final concentration of 1% in the assay medium.

(c) Fluorescence intensity of the fluorescein-labelled anti-(rat serum), purchased as the globulin fraction from Mercia Ltd., as a function of the protein concentration.
contained different amounts of antibodies, the antisera were tested against normal rat serum by the immunoelectrophoresis method. Fig. 3-14 shows that the anti-(rat serum) purchased from Mercia Ltd. produced more precipitation lines as compared to the lines formed by the antiserum obtained from Wellcome Reagents. This indicates that the amount of antibodies against rat serum protein that are present in the former antiserum is more than that in the latter. Hence one would suggest that the two antisera should show different activity towards our plasma membrane preparation.

The results presented in Fig.3-12 and 3-13 are consistent with this suggestion, and consequently Mercia anti-(rat serum) globulin was used in all further experiments.

From these results there would seem to be a considerable number of antigens common to rat serum and plasma membrane isolated from rat liver. Although plasma membranes used in these experiments were prepared from perfused livers, yet in any perfusion process a considerable amount of blood is left in the liver, so that absorbed serum proteins may contaminate the isolated membranes. The results presented earlier (section 3:3) show that washing the isolated plasma membrane successively with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0 removes serum and cytosol protein from our plasma membrane preparations. Therefore, to avoid the possibility that the results presented above are mainly due to material contaminating our plasma membrane
Immunoelectrophoresis plate, stained for proteins with Ponceau S, of rat serum against anti-(rat serum) obtained:
a) from Mercia Ltd.
b) from Wellcome Reagents Ltd.
In all cases an aliquot of rat serum was electrophoresed for 2 hours at about 10V/cm across the plate in 0.044 M-barbitone buffer, pH 8.6 at room temperature. The antisera was added immediately and allowed to diffuse for 48 hours at room temperature. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plate was stained for proteins.
preparation, the experiments were repeated using plasma membrane washed with 0.15 M-NaCl and 0.2 M-bicarbonate buffer. The results presented in Table 3-7 show that the proportion of anti-(rat serum)-binding materials removed from the membrane during the washing procedure is about the same as the proportion of the total membrane protein which is solubilized. The washed plasma membrane still retain about 70% of its anti-(rat serum) binding material.

Table 3-7: Percentage of material removed by washing the isolated plasma membrane successively with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0. The results are given as means ± standard deviation followed by number of observations (in parentheses).

<table>
<thead>
<tr>
<th>% of material recovered in</th>
<th>Protein</th>
<th>Anti-(rat serum)-binding material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.15 M-NaCl Wash</td>
<td>0.2 M-bicarbonate Wash</td>
</tr>
<tr>
<td>Protein</td>
<td>6.62 ± 1.1 (10)</td>
<td>27.5 ± 2.6 (10)</td>
</tr>
<tr>
<td>Anti-(rat serum)-binding</td>
<td></td>
<td>67.62 ± 1.3 (4)</td>
</tr>
<tr>
<td>material</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results are given as percentage of the fluorescence intensity of protein found in membrane stored in 0.15 M-NaCl at 4°C during the 48 hours required for the washing procedure. Anti-(rat serum)-binding material was determined on aliquots containing 0.7 mg membrane protein, washed with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0 as described in Table 3-3.

The washed membranes were incubated with 20 mg of the fluorescein-labelled anti-(rat serum) globulin for 30 min at 37°C as described in Fig. 3-13b.
From the experiments described above it was concluded that the antigenic components common to rat serum and liver plasma membrane are not an artifact due to the isolation procedure, but they are firmly associated with the membrane. Among these antigenic components may be proteins released in vivo from the liver cell membrane into the circulating fluid. In a preliminary experiment to identify the antigens common to rat serum and liver plasma membrane, anti-(rat serum) was absorbed extensively with plasma membrane washed with 0.15 M-NaCl and 0.2 M-bicarbonate buffer. Comparison of the immunoelectrophoresis pattern formed by rat serum against absorbed anti-(rat serum) with the pattern against unabsorbed anti-(rat serum) indicated that the antibodies removed by this absorption procedure mainly correspond to serum proteins which migrate in the β-globulin region (Fig. 3-15). Moreover, anti-(plasma membrane), raised against the membrane "core", i.e. plasma membrane washed with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, showed a considerable reaction when tested against rat serum (Fig. 3-16a). This was later used as a test for the strength of the anti-(plasma membrane) prepared. Immunelectrophoresis of rat serum against anti-(plasma membrane) using Agar No. 2 in 0.044 M barbitone buffer, pH 8.6, showed the presence of at least eight precipitation lines representing the common proteins. Four of these proteins were negatively charged, two were positively charged, besides two protein staying
Immunoelectrophoresis plate of rat serum against anti-(rat serum) and that absorbed with liver plasma membrane. (1) Whole anti-(rat serum). (2) Absorbed anti-(rat serum) with plasma membrane then concentrated up to the same original volume used before absorption. (3) Whole anti-(rat serum) diluted one to three. (4) Absorbed anti-(rat serum) diluted one to three. Electrophoresis was performed in 0.044 M-barbitone buffer, pH 8.6 for 2 hours at 10 V/cm across the plate. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plate was stained for proteins with Ponceau S.
at the origin (Fig. 3-16b). An attempt was then made to separate the proteins common to rat serum and liver plasma membrane by affinity chromatography on immobilised anti-(plasma membrane) as will be described in the following section.

(a) Ouchterlony double diffusion plate of rat serum (S) against anti-(plasma membrane) antiserum. Wells 1, 2, 3 and 4 contain antisera obtained from four different rabbits respectively. Well 5 contain anti-(plasma membrane) globulin pooled from rabbit 1 and 2.

(b) Immunelectrophoresis plate of rat serum against pooled anti-(plasma membrane) (A/PM) stained for proteins with Coomassie Brilliant blue R. For electrophoresis conditions see Fig 3-15.
Before the serum could be fractionated by affinity chromatography, it was necessary to immobilise the antibodies by linking them to a suitable matrix. Immobilised antibodies were prepared according to the method of Cuatrecasas et al. (1968) as described in Chapter 2 "Materials and Methods". Proteins not covalently linked to the cyanogen bromide activated Sepharose 4B were removed by washing exhaustively with 0.15 M-NaCl. When washing was complete the amount of coupled protein was determined as the difference between that recovered in the washings and that originally used. All preparations that were carried out resulted in the immobilisation of a considerable amount of protein from the antisera used and Table 3-8 shows that the amount bound was fully reproducible. The unreacted active groups of the activated Sepharose 4B were blocked off by washing the immunoadsorbent, i.e. Sepharose 4B-antibodies conjugate, with 50 mM Glycine-HCl buffer, pH 2.8 overnight at 4°C. The immunoadsorbent was then exposed to all eluents to which the immunoadsorbent-antigen complex is to be exposed.

To assess the merits of this procedure in retaining the biological activity of the antisera and to establish the exact
Table 3-8: Amount of antisera protein bound to the cyanogen bromide activated Sepharose 4B.

Sepharose 4B was activated by the method of Cuatrecasas et al. (1968) and incubated with the antisera in bicarbonate buffer pH 9.0, at 4°C for 24 hours. The slurry was poured into a 1.5 x 50 cm column, allowed to pack and washed exhaustively with 0.15 M-NaCl until no protein was detected in the washing, as measured either by Lowry method or as absorbion at 280 nm.

The results are presented as means ± standard deviation, with number of observations (in parentheses).

<table>
<thead>
<tr>
<th></th>
<th>Vol. of settled Sepharose 4B (ml)</th>
<th>Antisera protein incubated (mg)</th>
<th>Unbound protein recovered in 0.15 M-NaCl Wash (mg)</th>
<th>*Bound protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-(plasma membrane) antiserum</td>
<td>20</td>
<td>200</td>
<td>139 ± 2.5 (15)</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>200</td>
<td>49.6 ± 1.3 (25)</td>
<td>150.4</td>
</tr>
<tr>
<td>Anti-(cell cytosol) antiserum</td>
<td>20</td>
<td>100</td>
<td>38.9 ± 3.6 (3)</td>
<td>61.1</td>
</tr>
</tbody>
</table>

* The results in the fourth column are given as the difference between the amount of protein incubated (column two) and that given in the third column.
conditions for coupling and recovering specific antigens, cytosol and immobilised anti-(cytosol) were used. After incubation at 4°C for various periods of time the uncoupled cytosol proteins were washed off exhaustively with 0.15 M-NaCl. Analysis of protein in the washings showed that the amount of cytosol proteins coupled to the immobilised anti-(cytosol) increases as the incubation time was increased (Fig. 3-17a). The best results were obtained when the incubation time was extended to 24 hours. The available antibody binding sites thus appear to be saturated with the corresponding antigens. The results presented in Table 3-9 show that most of the cytosol enzyme activities that were assayed, were associated with the majority of the proteins that were coupled to the anti-(cytosol) conjugate.

To recover the proteins coupled to the immobilised anti-(cytosol), the immunoadsorbent-antigen complexes were exposed to different solutions, namely:

- 50 mM Glycine-HCl, pH 2.8
- 3 M Sodium thiocyanate, 50 mM phosphate, pH 6
- 1 M Propionic acid, pH 2.5
- 2.5 M Magnesium chloride.

The elution process was carried out, on separate columns, using 500 ml of each eluent at 4°C, and the eluted fractions were either dialysed against several changes of 0.15 M-NaCl (3 x 2 L; in case of magnesium chloride and thiocyanate) or neutralized to pH 7.0
a) Binding of liver cell cytosol to anti-(cell cytosol) conjugate as a function of time. Immobilised immunoglobulin containing about 60 mg protein was incubated with about 300 mg cytosol protein at 4 °C in 0.15 M-NaCl. The slurry was then packed in a 1.5 x 50 cm column and washed exhaustively with a 0.15 M-NaCl until no protein was detected in the washing. The amount of protein bound to the available antibodies was determined as the difference between the amount incubated and that recovered in the 0.15 M-NaCl washings.

b) Pattern obtained, by the 280 nm method, of cytosol proteins eluted by 2.5 M-MgCl ×—×; propionic acid○—○ Fractions obtained by eluting with propionic acid were neutralized to pH 7.0 before measurement.
Table 3-9: Percentage of protein and enzyme activities of liver cell cytosol bound to the immobilised anti-(cell cytosol) antibodies.

Immobilised anti-(cell cytosol) containing about 60 mg protein was incubated, for 24 hours at 4°C in 0.15 M-NaCl, with about 300 mg cytosol protein. The slurry was then packed in a 1.5 x 50 cm column and washed exhaustively with 0.15 M-NaCl until no protein was detected in the washings.

The results are given as means ± standard deviation followed by number of observations (in parentheses). The amount of cytosol protein and enzyme activities bound was determined as the difference between the amount incubated and that recovered in the 0.15 M-NaCl wash.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>% Recovered in 0.15 M-NaCl Wash</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>15 ± 2.3 (3)</td>
<td>85</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>12 ± 1.7 (3)</td>
<td>88</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>22.4 ± 2.6 (3)</td>
<td>87.6</td>
</tr>
<tr>
<td>Alkaline phosphodiesterase</td>
<td>14.5, 13.8</td>
<td>85.5, 86.2</td>
</tr>
</tbody>
</table>
(in case of propionic acid and glycine buffer). Analyses of Protein and enzymes in the eluted fractions showed that each of the four eluents has the ability to dissociate the antigen-antibody complex to a certain extent. The best results were obtained when 2.5 M-MgCl$_2$ was used as eluent (Table 3-10). Therefore, 2.5 M-MgCl$_2$ was afterwards used routinely as the eluting solution, and elution was carried out until no further protein was detected in the eluted fractions (Fig. 3-17b).

The results presented above indicate that the antibodies covalently bound to the Sepharose 4B retain their capacity to bind the corresponding antigens. The coupled antigens could be then detached by a method which did not destroy their enzyme activity.

To separate serum proteins common to rat liver plasma membrane, immobilised anti-(plasma membrane) was prepared by the method of Cuatrecasas et al. (1968) and treated exactly as the immobilised anti-(cytosol) (Table 3-8). In preliminary experiments, 3 ml rat serum containing 35 mg/ml protein was mixed with the immobilised anti-(plasma membrane) at 4°C for varying periods of time. The slurry then packed in a 1.5 x 50 cm column and the uncoupled serum proteins were washed off with 0.15 M-NaCl, and the protein content of the washings was determined. The difference between the amount of protein recovered in the washings and that originally incubated was considered as the amount of proteins that bound to the anti-(plasma membrane) antibodies. From these
Table 3-10: Percentage of protein and enzyme activities recovered with different eluents by affinity chromatography of liver cell cytosol on immobilised anti-(cytosol).

The results are given as means ± standard deviation followed by number of observations (in parentheses).

For the experimental conditions see Table 3.8. The results are presented as percentage of the total protein and enzyme activities in the cytosol incubated with the antiserum conjugate.

<table>
<thead>
<tr>
<th>% Recovered in</th>
<th>2.5 M-MgCl₂</th>
<th>1 M-Propionic acid, pH 2.5</th>
<th>3 M-Sodium thiocyanate 50 mM-phosphate pH 6.0</th>
<th>50 mM Glycine-HCl, pH 2.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>80 ± 2.6 (3)</td>
<td>45.5 ± 1.7 (3)</td>
<td>55 ± 3.1 (3)</td>
<td>38 ± 1.9 (3)</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>85.6 ± 1.7 (3)</td>
<td>51.6 ± 2.8 (3)</td>
<td>59 ± 4.9 (3)</td>
<td>52.7 ± 1.4 (3)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>83 ± 1.4 (3)</td>
<td>61 ± 2.7 (3)</td>
<td>67.8, 68.2</td>
<td>69.5, 68.1</td>
</tr>
<tr>
<td>Alkaline phosphodiesterase</td>
<td>75.8, 71.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
experiments, it was found that as the incubation time was increased, increasing amounts of serum proteins were bound to the immobilised antibodies. The available antibodies were found to bind all the common proteins in rat serum when the incubation time was extended up to about 24 hours. This time was afterwards chosen for routine assays (Fig. 3-18).

To determine the percentage of proteins in rat serum which are common to rat liver plasma membrane, about 100 mg rat serum protein was incubated with the immobilised anti-(plasma membrane) at 4°C for 24 hours, and the slurry then treated exactly as above. The results presented in Table 3-11 show that 4-5% of rat serum proteins are capable of binding to the anti-(plasma membrane) antibodies. However, enzymic analyses of the 0.15 M-NaCl wash showed that the serum enzymes that are of interest were mainly recovered in the washings. This indicates that those enzymes were incapable of binding to the anti-(plasma membrane) antibodies under these experimental conditions.

Later, the quantity of rat serum protein, that was incubated with the immobilised anti-(plasma membrane) antibodies, was increased gradually until all the available antibody binding sites appeared to be saturated. Material from rat serum bound to the immobilised antibodies was then eluted using a variety of agents exactly as described in the experiments with liver cell cytosol. The eluted fractions were, similarly, either dialysed against
Fig 3-18

Binding of rat serum protein to anti- (liver cell plasma membrane) conjugate as a function of time. Immobilised immunoglobulin containing about 60 mg anti- (plasma membrane) protein was incubated with about 100 mg rat serum protein at 4° C in 0.15 M-NaCl. The slurry was then packed in a 1.5 x 50 cm column and washed exhaustively with a 0.15 M-NaCl until no protein was detected in the washing. The amount of protein bound to the available anti- (plasma membrane) antibodies was determined as the difference between the amount incubated and that recovered in the 0.15 M-NaCl washing.
Table 3-11: Percentage of protein and enzyme activities of rat serum bound to the immobilised anti-(liver plasma membrane).

Immobilised anti-(plasma membrane) containing about 60 mg protein suspended in 0.15 M-NaCl was stirred with about 100 mg rat serum protein for 24 hours at 4°C. The slurry then packed in a 1.5 x 50 cm column and washed exhaustively with 0.15 M-NaCl until no protein was detected in the washings.

The results are given as means ± standard deviation followed by number of observations (in parentheses).

The amount of serum protein and enzyme activities bound was determined as the difference between the amount incubated and that recovered in the 0.15 M-NaCl wash.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>% Recovered in 0.15 M-NaCl Wash</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>95 ± 1.2 (8)</td>
<td>5</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>96.5 ± 1.7 (6)</td>
<td>3.5</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>102 ± 2.4 (4)</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline phosphodiesterase</td>
<td>103 ± 1.3 (4)</td>
<td>-</td>
</tr>
<tr>
<td>L-leucyl-β-naphthylamidase</td>
<td>91.5 ± 2.6 (4)</td>
<td>8.5</td>
</tr>
<tr>
<td>Ribonuclease, assayed at pH 7.8 with 5 mM-MgCl₂</td>
<td>90 ± 3.5 (3)</td>
<td>10</td>
</tr>
</tbody>
</table>
several changes of 0.15 M-NaCl or, in the case of propionic acid, simply neutralized to pH 7.0. Analyses of protein in the eluted fractions (Table 3-12) showed that the highest recovery was obtained when 2.5 M-MgCl₂ was used as the eluent. Enzymic activities could only be detected in the MgCl₂ eluate. These results are similar to those obtained in the trial experiments with cytosol and anti-(cytosol) i.e. in both cases the best results were obtained when 2.5 M-MgCl₂ was used. Therefore 2.5 M-MgCl₂ was afterwards used routinely as the eluting solution, and elution was carried out until no further protein was detected in the eluted fractions (Fig. 3-19).

The possibility that some proteins may bind non-specifically to the immobilised anti-(plasma membrane) antibodies, as a result of ionic interaction, was examined by washing the column containing the immunoadsorbent and 'bound' antigen with 0.5 M-NaCl prior to eluting it with 2.5 M-MgCl₂. Analyses of protein in the 0.5 M-NaCl eluate showed that a considerable amount of material was eluted by this process. An additional amount of serum protein was also eluted when 2.5 M-MgCl₂ was used as an eluent. However, gradient polyacrylamide gel electro-phoresis of the concentrated material recovered in the two eluate showed that both fractions contain identical proteins (Fig. 3-20). This suggests that there was hardly any non-specific binding, and the material recovered in both 0.5 M-NaCl and 2.5 M-MgCl₂
Table 3-12: Percentage of protein and enzyme activities recovered with different eluents by affinity chromatography of rat serum on immobilised anti-(plasma membrane).

The results are given as means ± standard deviation followed by number of observations (in parentheses).

For experimental conditions see Table 3-10. The results are given as percentage of the total protein and enzyme activities in the serum incubated with the anti-(plasma membrane) conjugate.

<table>
<thead>
<tr>
<th>% Recovered in</th>
<th>2.5 M-MgCl₂</th>
<th>1 M-Propionic acid, pH 2.5</th>
<th>3 M-Sodium thiocyanate, pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>3.5 ± 1.1 (8)</td>
<td>2.1 ± 0.8 (8)</td>
<td>1.5 ± 0.7 (3)</td>
</tr>
<tr>
<td>5′-Nucleotidase</td>
<td>&lt; 0.5</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>&lt; 0.5</td>
<td>&lt; 0.1</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Alkaline phosphodiesterase</td>
<td>&lt; 0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-leucyl-β-naphthylamidase</td>
<td>7.3 ± 1.2 (4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Riconuclease assayed at pH 7.8 with 5 mM-MgCl₂</td>
<td>7.5 ± 1.6 (3)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Pattern obtained by 280 nm method of serum proteins eluted by 2.5 M-MgCl\( \times \cdots \times \), 1M-propionic acid\(--\circ--\). Fractions obtained by elution with propionic acid were neutralized to pH7.0 before measurement.
Gradient polyacrylamide gel electrophoresis (Universal Scientific, London) of rat serum and of a concentrated sample of serum proteins homologous to rat liver plasma membrane. The latter was isolated by binding serum proteins to immobilised anti-(plasma membrane) and eluting with 0.5 M-NaCl (A) and 2.5 M-MgCl₂ (B). Electrophoresis was performed in tris-EDTA-boric acid buffer, pH 8.4 (Margolis and Kenrick, 1967) for 15 hours after which the gel was stained for proteins with Coomassie Brilliant blue R.
eluate represents serum protein specifically bound to the anti-(plasma membrane) conjugate. When 2.5 M-MgCl$_2$ was used as eluent, without pre-elution with 0.5 M-NaCl, the amount of protein recovered was almost equivalent to that in the two step elution process. Therefore, it was concluded that the material eluted by 2.5 M-MgCl$_2$ represents only serum proteins bound specifically to the anti-(plasma membrane) antibodies.

Electrophoresis of a concentrate of the material recovered in the 2.5 M-MgCl$_2$ eluate on polyacrylamide gel gave eight bands when the gel was stained for protein (Fig. 3-21a). Three of these bands were identified as glycoprotein by staining the gel for carbohydrate with periodic acid-Schiff reagent (Fig. 3-21b). These three bands mainly correspond to serum glycoproteins which migrate to the $\alpha_1$ and $\beta$-globulin region (Fig. 3-21c). When the gel was stained for L-leucyl-$\beta$-naphthylamidase activity by the method of Gomori (1954), the protein that migrated to the $\alpha_1$ position was found to be enzymatically active (Fig. 3-21d). Immunoelectrophoresis of the 2.5 M-MgCl$_2$ eluate against anti-(rat serum) and anti-(plasma membrane) antisera showed the presence of at least seven precipitation lines which were identical for the two antisera (Fig. 3-22). A few other lines were only detected against anti-(rat serum). These results would also support the suggestion that the material eluted by 2.5 M-MgCl$_2$ represent only serum proteins specifically bound to our anti-(plasma membrane) conjugate preparation.
Polyacrylamide-gel electrophoresis in 0.05 M Tris-0.4 M glycine buffer, pH 8.3 of rat serum and of a concentrated sample of the material bound to the immobilised anti-(plasma membrane) and recovered in the 2.5 M-MgCl₂ eluate (ES). Electrophoresis was performed for 2 hours at room temperature using 5 mA/tube.

a) Polyacrylamide-gel electrophoresis of (ES) stained for proteins with Ponceau S. Eight protein bands are detected.

b) Same as in (a) stained for carbohydrate by the periodate-Schiff reagent. Three bands were detected.

c) Polyacrylamide-gel electrophoresis of rat serum stained for carbohydrate by the periodate-Schiff reagent. Three main glycoprotein bands are detected.

d) Polyacrylamide-gel electrophoresis of (ES) stained for L-leucyl-β-naphthylamidase activity by the method of Gomori (1954). The protein that migrate to the $\alpha_1$ position was found to be enzymatically active.
Fig 3-21

(a)

(b)

(c)

(d)

Origin

Origin
Immunoelectrophoresis, against anti-(rat serum) (A/S) and anti-(plasma membrane) (A/PM) antisera, of rat serum and a concentrated sample of serum proteins homologous to rat liver plasma membrane (ES). The latter was isolated by binding serum proteins to immobilised anti-(plasma membrane) and eluting with 2.5 M-MgCl₂. In all cases an aliquot containing about 14 mg protein was electrophoresed for 2 hours at about 10 V/cm across the plate in 0.044 M-barbitone buffer, pH 8.6 at room temperature. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plates were stained for proteins with Coomassie Brilliant blue R.
To test that all serum proteins that can react with anti-(plasma membrane) are bound to the column and can be eluted by 2.5 M-MgCl₂, rat serum was electrophoresed parallel with the concentrated material eluted by 2.5 M-MgCl₂ and allowed to diffuse against anti-(plasma membrane). Examination of Fig.3-22 indicated that serum and the eluted serum proteins gave almost the same immunoelectrophoresis pattern. The only missing protein from the eluted serum was that which is responsible for the formation of line (1) in the normal serum pattern. This could be explained by either of the following possibilities:-

i) The antibodies against this particular protein are not covalently bonded to the activated Sepharose under our coupling conditions.

ii) There is either some sort of degradation of this type of serum protein as a result of the elution procedure or the 2.5 M-MgCl₂ is not effective enough to elute this particular protein.

The material eluted by 2.5 M-MgCl₂ was further examined by comparing it with the material extracted from the isolated liver plasma membrane by 0.15 M-NaCl and 0.2 M-bicarbonate buffer. When the three concentrated fractions were allowed to diffuse against anti-(rat serum) (Fig. 3-23a), it was found that all the lines formed by both 0.15 M-NaCl and 0.2 M-bicarbonate are continuous with those formed by the eluted serum proteins.
Ouchterlony double diffusion plates of rat serum (S), a concentrated sample of (ES) (see Fig 3-22), 0.15 M-NaCl (SW) and 0.2M-bicarbonate (BW) extracts against (a) anti-(rat serum) (A/S), (b) anti-(liver plasma membrane) (A/PM) antisera. In all cases an aliquot containing about 14 mg protein was allowed to diffuse against the antisera for 48 hours at room temperature. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plates were stained for proteins with Ponceau S.
The major lines formed by eluted serum proteins are not detectable in either 0.15 M-NaCl or 0.2 M-bicarbonate buffer. This indicated that serum-like proteins extracted by 0.15 M-NaCl and 0.2 M-bicarbonate buffer from the isolated plasma membrane are not among the two or three major constituents of the proteins recovered in the 2.5 M-MgCl₂ eluted serum. The point of interest in Fig. 3-23b is the pattern formed against anti-(plasma membrane) by both 0.15 M-NaCl and 0.2 M-bicarbonate buffer. This shows that the major plasma membrane proteins extracted by NaCl and NaHCO₃ are not identical with the major plasma membrane-like proteins found in rat serum and the eluted serum. Minor constituents are the only identical components found in all these fractions which are responsible for the formation of the continuous lines near to the central well. The proteins recovered in the eluted serum, the 0.15 M-NaCl and 0.2 M-bicarbonate buffer washes were compared with the sodium deoxycholate + Lubrol W-solubilized plasma membrane. Fig. 3-24 shows, as expected, that when allowed to diffuse against anti-(plasma membrane), the eluted serum, the 0.15 M-NaCl and 0.2 M-bicarbonate washes, form lines that are continuous with those formed by the solubilized plasma membrane. The major lines formed by solubilized plasma membrane are not detectable among the lines formed by any of the other fractions. The results shown in Fig. 3-24 also support the suggestion that the plasma membrane-like proteins recovered in the eluted serum are not identical with those extracted by 0.15 M-NaCl and 0.2 M-NaHCO₃.
Ouchterlony double diffusion plate of rat serum (S), a concentrated sample of (ES) (see Fig 3-22), 0.15M-NaCl (SW), 0.2M-bicarbonate (BW) extracts and of the solubilized plasma membrane (SPM) against anti-(plasma membrane) antiserum (A/PM). The plasma membrane was solubilized in sodium deoxycholate + Lubrol W as described in section (2:6). In all cases an aliquot containing about 14 mg protein was allowed to diffuse against the antiserum for 48 hours at room temperature. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plate was stained for proteins with Ponceau S.
Furthermore, such proteins are not major components of the isolated plasma membrane.

The results presented above demonstrate the presence of antigenic groups that are common to rat serum and liver plasma membrane. The common groups represent about 4% of rat serum proteins which can be recovered in the 2.5 M-MgCl₂ eluate. Enzymic analysis of the eluted fraction indicated that the common proteins do not include the most commonly used marker enzymes of liver plasma membrane. Some of these common components are glycoproteins which could be either a transient component temporarily complexed with the membrane, or they are part of structural proteins of the membrane. Examination of these two possibilities was performed using labelled fucose as a precursor of glycoprotein. The results of these experiments will be described later.
As described earlier, part of the plasma membrane of liver cell is fragmented to vesicles during homogenisation. These vesicles are mainly recovered in the microsomal fraction. The distribution of the most commonly used plasma membrane marker enzymes among plasma membrane fragments of microsomal size was examined using isopycnic flotation in a linear sucrose density gradient in a B XIV zonal rotor as described in section 2:5b. Briefly, the microsomal fraction separated from homogenates of perfused livers was resuspended in 2 M sucrose buffered with 5 mM Tris-HCl, pH 8 and introduced at the dense end of a linear gradient ranging in density from 1.077 to 1.22 g/ml. Flotation was carried out at 45000 revs/min for 16 hours and 20 ml fractions were collected.

After flotation of a suspended microsomal fraction through a linear sucrose density gradient, analysis of the distribution of protein shows that the material has been separated into two zones banding at densities of about 1.165 and 1.22 g/ml (Fig. 3.25a). The first zone was identified as smooth endoplasmic reticulum fragments as indicated by glucose 6-phosphatase activity. The second protein peak corresponds to the sample region. Rough endoplasmic reticulum as indicated by RNA was located between the smooth endoplasmic reticulum and the sample region (Fig. 3.25a). Enzyme analysis of the fractions recovered
Protein and enzyme distribution after flotation of a rat liver microsomal preparation for 16 hours at 45,000 rev/min in a B XIV zonal rotor. The microsomal fraction from 20 g liver was resuspended in 30 ml of 2 M-sucrose and introduced at the dense end of a linear sucrose density gradient ranging from a density of 1.077 to a density of 1.22 g/ml. (a) --- ---, protein; --- ---, density; □ --- □, glucose 6-phosphatase (G6Pase); v---v, RNA. (b) o--o, 5'-nucleotidase (AMPase); □---□, alkaline phosphodiesterase (Alk. PDase, measured with bis-p-nitrophenyl phosphate as substrate); v---v, L-leucyl-β-naphthylamidase (LNase). Other plasma membrane enzymes, namely alkaline p-nitrophenyl phosphatase, ADPase, ATPase were all coincident in distribution with L-leucyl-β-naphthylamidase activity.
after flotation showed that 5'-nucleotidase was concentrated in a zone centred at a density of 1.14 g/ml (Fig. 3.25b). The proportion of 5'-nucleotidase activity found in this region was about 55% of the total microsomal activity. The rest of this enzyme activity, together with other plasma membrane enzymes, namely alkaline p-nitrophenyl phosphatase, alkaline phosphodiesterase, L-leucyl-β-naphthyl-amidase, ADPase and ATPase activities were all associated with material banding at a density of about 1.165 g/ml, coincident with smooth endoplasmic reticulum fragments indicated by glucose 6-phosphatase activity. Little activity of these six "plasma membrane" enzymes was found in the region of rough endoplasmic reticulum fragments (Fig. 3.25b).

To ascertain whether the differences in pattern between 5'-nucleotidase and other enzymes, which are all predominantly located in the plasma membrane (see introduction p.30) were due to variation in the permeability of the membrane vesicles, the enzyme assays were repeated in the presence of 1% Triton X-100. The enzyme distribution pattern obtained in the presence of detergent was found to be identical with that shown in Fig. 3.25b in the absence of detergent. These results demonstrate that 5'-nucleotidase does not have the same distribution among the vesicles present in the microsomal fraction as compared to five other enzymes also reported to be located in the plasma membrane. This suggests that the low density 5'-nucleotidase may have originated not from the plasma membrane, but from some other cell structures which, upon homogenisation, produce fragments
To ascertain whether the low density 5'-nucleotidase is the same enzyme as that found in the dense vesicles, histochemical staining for 5'-nucleotidase was performed on the immunoprecipitation lines formed by the solubilised fractions against our anti-(liver plasma membrane) antiserum.

Vesicles were collected from the pooled fractions (see Fig. 3.25) by spinning at 40,000 revs/min for 90 minutes after dilution with an equal volume of distilled water (see Table 2.1). These pellets and the pellets of plasma membrane sheets, separated from the crude nuclear fraction as described in section 2:5a, were solubilized by resuspension in N-dodecylsarcosinate Tris-HCl, pH 7.8 (Evans and Gurd, 1972). In the case of the membrane sheets a deoxycholate extract was also included. An aliquot of the suspension was allowed to diffuse against our anti-(liver plasma membrane) antiserum in an Ouchterlony double diffusion plate as described in section 2:11. Histochemical staining of the wet gel for 5'-nucleotidase with AMP as substrate (Fig. 3.26) showed that the enzyme that was associated with all microsomal subfractions is immunologically invariant. Furthermore, this enzyme is also identical with that associated with liver plasma membrane sheets isolated from the crude nuclear fraction.

The protein precipitation lines formed by the microsomal subfractions and their relation to that formed by the membrane sheets will be considered later.

As discussed later, these results together with observations by other workers indicated that the subfractions separated by flotation
Fig. 3.26

Comparison of the reaction of 5'-nucleotidase in microsomal subfractions and plasma membrane sheets against anti-(plasma membrane) antiserum. Out of the fractions collected from flotation of the microsomes, 7 ml aliquots were combined as follows:

- High 5'-nucleotidase and low glucose 6-phosphatase Region A
- 5'-nucleotidase peak Region B
- High 5'-nucleotidase and low glucose 6-phosphatase Region C and Region D
- First glucose 6-phosphatase peak Region E
- High glucose 6-phosphatase and low 5'-nucleotidase Region F
- Second glucose 6-phosphatase peak Region H

The regions are given in Fig. 3.25.

In all cases the membranes were solubilized in 0.5 ml of 4% (w/v) Na-dodecylsarcosinate - 2.1% (w/v) Tris-HCl, pH 7.8. A deoxycholate extract of the plasma membrane sheets was also included. An aliquot of the membrane suspension was allowed to diffuse against anti-(plasma membrane) antiserum. Precipitation was allowed to proceed for 48 hours at room temperature and the unprecipitated proteins were washed off with 2.5% NaCl. The wet plates were stained for 5'-nucleotidase activity with AMP as substrate by the method of El-Aaser et al. (1973).
In the outside wells an aliquot of the deoxycholate extract of the material obtained from: 1) region A; 2) region B; 3) region C; 4) region D; 5) region E; 6) region F; 7) region G; 8) region H; 9) plasma membrane sheets isolated by an A XII zonal run; whereas 10) is the sarcosyl extract of the plasma membrane sheets isolated by an A XII run.
of the microsomes are actually plasma membrane fragments of microsomal size. It is known that in the liver cell plasma membrane there are two distinct, metabolically active, areas - the sinusoidal surface and the biliary surface. It has been suggested, though without definite proof, that the plasma membrane vesicles recovered in the microsomal fraction are derived largely from the sinusoidal surface of the liver cell (Hinton et al., 1971). To test this hypothesis an attempt was made to label the sinusoidal-surface of the liver cell by perfusing the liver in situ with a fluorescent dye which would bind to the membrane surface and act as a tracer during cell fractionation. The results of these experiments are described below.
Attempts were made to label the membranes facing on to liver sinusoids with 4-acetamide-4'-iso-thiocyanato-stilbene-2,2'-disulphonic acid disodium salt (SITS). The liver was perfused in situ through the portal vein with, in succession, warm (~37°C) 0.25 M sucrose, 50 μM SITS and finally with 0.25 M sucrose solution as described in section 2:5c. Small pieces were taken from different parts of the liver for morphological studies and either fixed immediately with formalin or kept in ice-cold 0.25 M sucrose.

Fluorescence microscopic examination of thin sections of the liver, so perfused, showed the blue fluorescence of SITS throughout the portal vein and in the terminal branches of the veins. Fluorescence was observed in what appeared to be the sinusoids (Fig. 3-27). Equal fluorescence was found in fixed or unfixed tissue. No other recognisable fluorescent structures could be seen with the fluorescence microscope. This suggested that the SITS is specifically labelling, in situ, the blood vessels and the sinusoidal-facing surface of the liver cell. Hence, it seemed that bound SITS could be used as a marker for sinusoidal membrane fragments in experiments such as those described in sections 2:5a and b.
Fluorescence micrograph of frozen sections of rat liver perfused in situ with SITS. The blue fluorescence of SITS was observed in the portal vein (PV) and sinusoids (S) as indicated, not in the bile-canalicular faces of the plasma membrane.

The liver of rat anaesthetized with ether, was perfused through the portal vein with warm, about $37^\circ$, 0.25 M sucrose, 5 mM Tris-HCl, pH 8.0 until completely blanched. It was then perfused with approximately 30 ml of a warm and freshly prepared solution of 50 μM SITS in 0.25 sucrose, pH 8.0. A further perfusion with 0.25 M sucrose ensured that the unbound SITS was completely washed out of the liver. Small pieces were taken from different parts of the liver and were either fixed with formalin, or kept in ice-cold 0.25 M sucrose. Thin sections were cut (about 5-6 μm) and were examined using the Leitz fluorescence microscope with a vertical illuminator.
The crude nuclear fraction and the microsomal fraction obtained from livers perfused with SITS were fractionated in AXII and B XIV zonal rotors as described in section 2:5. The fluorescence intensity at 483 nm with excitation at 350 nm in the fractions collected after centrifugation was measured in the presence of 14% KOH (Knauf and Rothestein, 1971). Fluorescence measurements on the fractions recovered from the AXII zonal run showed that the slow-sedimenting material, particles in the mitochondrial region and the nuclei and aggregated material all possessed a high fluorescence as compared to the plasma membrane region (Fig. 3-28a). In the B XIV run, however, it appears that the fluorescence intensity pattern resembles that of 5'-nucleotidase more than any of the other plasma membrane enzymes (Fig. 3-28b). To ascertain whether such a fluorescence distribution pattern is due to the bound SITS or to some endogenous fluorescent material, the fluorescence intensity of a "blank run" was measured. This involved measuring the fluorescence of fractions recovered from centrifugation of a crude nuclear and microsomal fractions of liver perfused with sucrose without SITS.

At this stage of the investigation fluorescence measurements on these fractions revealed that very little endogenous fluorescence is present in the "blank runs". As a result, it was thought that probably our perfusion technique is not effective enough to remove traces of free SITS left in the blood vessels, which after homogenisation labelled the membrane proteins randomly. Since SITS is capable of binding to the membrane protein via an amino group, therefore, an attempt was
Distribution pattern of protein, enzymes and fluorescence from liver perfused in situ with SITS, obtained after centrifugation of:

a) The crude nuclear fraction, for 50 minutes at 3700 revs/min in an A XII zonal rotor. --- , density; ▲ , fluorescence; + -- , succinate dehydrogenase (SuccDase); O --- O, 5'-nucleotidase (AMPase).

b) The microsomal fraction, for 16 hours at 4500 revs/min in a B XIV zonal rotor. --- , density; ▲ , fluorescence; □ --- □, glucose 6-phosphatase (G6Pase); O --- O, 5'-nucleotidase (AMPase).

Prior to measurement of the fluorescence, the collected fractions were digested in 14% KOH. To ensure complete digestion, incubation at 45°C was carried out for between 24 and 48 hours until a clear solution was obtained. In some instances sonication was necessary to obtain complete solubilization of all membranes. The fluorescence of the digest was measured at 483 nm with excitation at 350 nm (Knauf and Rothstein, 1971).
made to improve the perfusion technique by flushing the liver with ethanolamine after perfusing with SITS. The fluorescence patterns obtained after such modification were found to be almost the same as that shown in Fig. 3.28. This would indicate that there was hardly any free SITS left after perfusion.

On repeating the experiments described above a problem emerged with the reproducibility of the fluorescence intensity measured in the fractions of the "labelled runs" and the "blank runs". This suggested that incomplete solubilisation of the membranes might be part of the problem. In our experiments we could not achieve complete solubilisation of the membrane even after sonication and incubation at 45°C in the presence of 14% KOH. However, during preparation of this thesis it was realised that the major problem is the calibration of the spectrofluorimeter. Hence, a few experiments were repeated and the best results were obtained when the fluorescence intensities of the fractions of a "labelled run" and that of a "blank run" were measured at the same time. Such measurements revealed that the particles from the crude nuclear fraction separated in the A XII run possess a considerable endogenous fluorescence. The distribution pattern of the fluorescence intensity appeared very similar to that observed in a "labelled run" (Fig. 3.29a). However, there was a considerable difference in the total amount of fluorescence present in each fraction of the two runs. Hence, it was thought that, the best way to obtain the exact pattern of the fluorescence intensity of the bound SITS is to subtract the endogenous fluorescence from
Fig 3-29

a) Distribution pattern of protein and fluorescence obtained after centrifugation for 50 minutes at 3700 revs/min in an AXII zonal rotor, of a crude nuclear fraction, obtained from livers perfused with a warm 0.25 M sucrose without SITS. —— density; ——— protein; —— fluorescence. For fluorescence measurements see Fig 3-28.

b) Fluorescence distribution pattern obtained by subtracting the fluorescence of the fractions of "blank run" shown in (a) from that of the fractions of the "labelled run" shown in Fig 3-28 a.
that measured in the fractions of the labelled run after correcting for the amount of protein present in each fraction. Such correction renders the fluorescence distribution pattern, especially from the A XII run, more easy to interpret. A distinct fluorescent peak was found in the beginning of the gradient coinciding with the slow sedimenting microsomes, in the plasma membrane sheets and in the nuclei and aggregated material at the end of the gradient (Fig. 3.29b). Peaks of fluorescence appeared also to be present between the plasma membrane sheets and the nuclei corresponding to particles with densities of 1.186 and 1.196 g/ml. It is interesting that these are the same densities as those reported by Hinton et al. (1970) for the two peaks of red blood cells found in similar runs. The apparent peaks in tubes 13 and 15, on the other hand, probably have no significance. The endogenous fluorescence of the mitochondria is exceptionally high and the corrections are correspondingly inaccurate.

In the B XIV zonal run, the endogenous fluorescence of the fractions was about 2/3rd. of that found in experimental tubes and showed an irregular pattern (Fig. 3.30a). Hence, at this stage of preparation of this thesis unfortunately no more time could be spent to investigate the reasons for this irregular endogenous fluorescence pattern. Hence, an approximation was made in which the average fluorescence intensity of the fractions of the "blank run" was subtracted from that measured on the fractions of the labelled run. The pattern obtained after such correction (Fig. 3.30b) shows that the fluorescence is mainly concentrated in the material banding at
**Fig. 3.30**

a) Distribution pattern of protein and endogenous fluorescence after flotation of the microsomal fraction through a linear density gradient in a B XIV zonal rotor. The microsomes were obtained from livers perfused with 0.25 M sucrose without SITS. --- , density; --- , protein; ▲, fluorescence. For fluorescence measurements see Fig. 3.28.

b) Corrected fluorescence distribution obtained by subtracting the average fluorescence intensity of the fractions of the "blank run" shown in (a) from that of the fractions of the "labelled run" shown in Fig. 3.28b. The distributions of glucose 6-phosphatase and 5'-nucleotidase are included as markers for the low and high density vesicles respectively.
Fig. 3.30
density of about 1.14 g/ml.

In spite of all the problems of measuring the fluorescence intensity of the "labelled" and the "blank" runs, these results indicate that the sinusoidal-facing surfaces of the liver plasma membrane, labelled in situ with SITS, are mainly recovered in the microsomal fraction. The subfraction of microsomal membranes banding at a density of about 1.14 g/ml appeared to contain a high proportion of fragments from sinusoidal-facing surface. Relatively little of the labelled surface is recovered with the plasma membrane-rich region isolated from the crude nuclear fraction by an AXII zonal run.
Earlier in this chapter it was demonstrated that proteins which are common to liver plasma membrane and rat serum could be isolated by affinity chromatography of rat serum on immobilised anti-(liver plasma membrane) antiserum. At least some of these common proteins were shown to be glycoproteins. These results encouraged us to investigate the release of glycoproteins from the liver cell plasma membrane to the serum, especially in view of the results presented by Riordan et al. (1974). These authors showed that labelled fucose was incorporated into liver cell plasma membrane in two distinct phases which they interpreted as reflecting incorporation into two groups of proteins. The rapid turnover of the first group suggests that they are secretory glycoproteins temporarily associated with the membrane. The second group would then presumably be membrane glycoproteins.

In our investigation, two experiments were performed in which L-$^{14}$C fucose was used as a precursor for glycoproteins. In one case the animals were killed 15 minutes after fucose injection, whilst in the second experiment the incorporation time was extended to 90 minutes. The livers were fractionated by zonal centrifugation and the serum by affinity chromatography following the procedure described in section 2.5d. Fractions from the B XIV zonal rotor were pooled as indicated in Fig. 3.33.
The plasma membrane-rich fraction separated from the crude nuclear fraction was further purified by flotation from a sucrose solution of density 1.19 g/ml as described in section 2.5a. The pellets obtained after spinning the pooled fractions from the B XIV rotor at 40000 revs/min (see Materials and Methods, Table 2.1) for 90 minutes, and the floated plasma membrane, were washed successively with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0, as described in sections 2.5a and 2.6. The washed pellets were resuspended in 0.15 M-NaCl and the radioactivity was measured. The seromucoids present in the salt extracts and in the washed membrane pellets were separated by the method of Sturgess et al. (1972) as described in section 2.5d.

After 15 minutes of fucose incorporation, fractionation of the serum on an immobilised anti-(liver plasma membrane) column showed a significant incorporation of radioactivity into proteins capable of binding to our anti-(plasma membrane) conjugate. The amount of the radioactively labelled proteins bound to the anti-(plasma membrane) conjugate was increased further when the incorporation time was 90 minutes (Table 3.13). More incorporation was detected after 90 minutes of fucose injection when serum was fractionated into acid-precipitable glycoproteins by the method of Sturgess et al. (1972). However, the percentage radioactivity of the labelled serum proteins that are capable of binding to our anti-(plasma membrane) conjugate decreased as the incorporation time was increased. Similar observations
Table 3.13: Recovery of $^{14}$C-fucose in perchloric acid-soluble and -insoluble serum glycoproteins and in glycoproteins binding to anti-(plasma membrane) antiserum in the fractionation of rat serum by affinity chromatography.

The results in the fourth column are presented as percentage of the total radioactivity recovered in serum glycoproteins.

<table>
<thead>
<tr>
<th>Incorporation time</th>
<th>Counts/min recovered in*</th>
<th>% Radioactivity recovered in the eluted serum fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCA-soluble</td>
<td>PCA-insoluble</td>
</tr>
<tr>
<td>15 minutes</td>
<td>28637</td>
<td>286236</td>
</tr>
<tr>
<td>90 minutes</td>
<td>76187</td>
<td>1592351</td>
</tr>
</tbody>
</table>

* In each case 4.2 ml rat serum was fractionated either by affinity chromatography on an immobilised anti-(liver plasma membrane) antiserum or into perchloric acid-soluble and-insoluble glycoproteins by the method of Sturgess et al. (1972). Radioactivity in the 2.5 M-MgCl$_2$ eluted serum fraction was measured with the aid of Lissapol-Scintillant and the results are presented as counts/min in a fraction containing 10 mg proteins, whilst that in the acid-precipitable serum glycoproteins was measured using Tritosol-Scintillant.
were deduced by examination of the radioautograms presented in Fig. 3.31. The radioactively labelled precipitation lines, formed by the material eluted from the column, against anti-(plasma membrane) antiserum were more defined after 15 minutes of fucose incorporation. This would suggest that serum glycoproteins that are common to liver plasma membrane are among the rapidly labelled proteins.

Measurement of the radioactivity in the crude nuclear fraction as subfractionated in the A XII zonal rotor showed that after both periods of fucose incorporation the label is concentrated in three bands coincident with the three 5'-nucleotidase bands (Fig. 3.32). Those three bands correspond to the microsomes (region 2), plasma membrane sheets (region 4) and nuclei contaminated with aggregated material (region 5) (see Fig. 3.1a, regions 2, 4 and 5).

As described earlier in this section, fractionation of the microsomes in a linear sucrose density gradient resulted in the separation of a distinct fraction of low density vesicles highly enriched in 5'-nucleotidase. Other plasma membrane enzymes and the remaining part of the 5'-nucleotidase activity are associated with material banding at a density similar to that of the smooth endoplasmic reticulum as indicated by glucose 6-phosphatase. Radioactivity measurements on the fractions showed that after neither period of fucose incorporation did the labelling pattern coincide exactly with any of the enzyme distribution patterns. Fig. 3.33a shows that after 15 minutes of fucose incorporation the radioactivity pattern is widely spread but appears to coincide with the 5'-nucleotidase peak banding at a density of about 1.14 rather than with
Radioautograms of the immunoelectrophoresis plates, against anti-(rat serum) (A/S) and anti-(plasma membrane) (A/PM) antisera, of rat serum (S) and a concentrated sample of the material bound to the immobilised anti-(plasma membrane) and eluted by 2.5 M-MgCl$_2$ (ES).

In all cases an aliquot containing about 14 mg protein was electrophoresed for 2 hours at about 10 V/cm across the plate in 0.044 M-barbitone buffer at room temperature. The antisera were added immediately. Diffusion and precipitation was allowed to proceed for 48 hours at room temperature. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plates were left, in the dark, in contact with an X-ray film for about three weeks.

a) after 15 minutes of fucose incorporation, b) after 90 minutes of fucose incorporation. c) Same as (b) but stained for proteins with Coomassie Brilliant blue R.
Distribution of protein, radioactivity and marker enzymes after centrifugation of a crude nuclear fraction for 50 min, at 3700 revs/min in an AXII zonal rotor.

(a) after 15 minutes of fucose incorporation, ———— density; ————, protein; ○○○, 5'-nucleotidase; □□□□□, glucose 6-phosphatase; ▼▼▼▼▼, radioactivity.

(b) after 90 minutes of fucose incorporation. Symbols are the same as in (a)
Distribution of enzymes and radioactivity after flotation of a rat liver microsomal fraction for 16 hours at 45000 revs/min in a B-XIV zonal rotor.

a) after 15 minutes of fucose incorporation, --- density; --- protein; o--o, 5'-nucleotidase, □----□, glucose 6-phosphatase; v→v, radioactivity.

b) after 90 minutes of fucose incorporation. Symbols are the same as in (a).

For experimental details see Fig 3-26.
the other plasma membrane enzymes. The measured radioactivity was found to be mainly due to high molecular weight glycoproteins, only 1/3rd of the radioactivity being attributable to material soluble in trichloroacetic acid (Table 3.14). After 90 minutes of fucose incorporation the radioactivity is found in both low density vesicles and those banding with the smooth endoplasmic reticulum (Fig. 3.33b). Comparison of Figs. 3.33a and 3.33b shows a considerable increase in radioactivity of the fractions coinciding with the smooth endoplasmic reticulum, whilst the radioactivity in the low density vesicles in decreased.

The results presented so far show that after both periods of fucose incorporation a considerably greater proportion of radioactivity was found in the microsomal subfractions than in the sheets of plasma membrane separated from the crude nuclear fraction. However, the relatively low activity in the plasma membrane fraction isolated from the crude nuclear fraction was substantially increased after 90 minutes of incorporation, whilst the activity in the microsomal subfractions decreased (Table 3.15).

Removing loosely bound proteins and the vesicle contents from the microsomal subfractions, obtained from the B XIV zonal run, by extraction with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0 does not affect the overall radioactivity pattern, but it does appear that after a short incorporation time more radioactivity in the form of loosely bound material is washed off from the microsomal subfractions than is removed in the case of the long period of incorporation. Accordingly, the washed pellets showed more radioactivity after
Table 3.14: Radioactivity recovered in the trichloroacetic acid (TCA) insoluble proteins of certain fractions selected across the B XIV zonal run after 15 minutes of fucose incorporation.

The results are expressed as counts/minute in a 0.5 ml aliquot of each of the fractions before and after precipitation with the acid.

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Counts/min as collected from the zonal run</th>
<th>Counts/min in the* TCA-insoluble fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>983</td>
<td>778</td>
</tr>
<tr>
<td>13</td>
<td>1196</td>
<td>861</td>
</tr>
<tr>
<td>15</td>
<td>1189</td>
<td>605</td>
</tr>
<tr>
<td>30</td>
<td>1257</td>
<td>551</td>
</tr>
</tbody>
</table>

* In each case an aliquot of the fraction was mixed with an equal volume of cold 10% TCA and stored in ice for 20 minutes. The precipitated proteins were collected on glass fibre filters. The filters were washed with ethyl alcohol and dried under an I.R. lamp. The dry filters were placed into scintillation vials and counted as described by Scherrer (1969).
Table 3.15: Radioactivity peaks found, after 15 and 90 minutes of fucose incorporation, in the fractions collected after centrifugation of the crude nuclear and microsomal fractions in an A XII and B XIV zonal rotor respectively.

The results are presented as counts/minute in 0.5 ml aliquot of each of the fractions counted in Lissapol-Scintillant.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Counts/min after 15 min of incorporation</th>
<th>Counts/min after 90 min of incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A XII run</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low sedimenting material (peak tube of region 1, Fig. 3.32)</td>
<td>1662</td>
<td>1062</td>
</tr>
<tr>
<td>plasma membrane sheets (peak tube of region 4, Fig. 3.32)</td>
<td>72</td>
<td>190</td>
</tr>
<tr>
<td>Nuclei and aggregate (peak tube of region 5, Fig. 3.32)</td>
<td>101</td>
<td>269</td>
</tr>
<tr>
<td><strong>B XIV run</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st radioactivity peak (Fig. 3.33)</td>
<td>1261</td>
<td>756</td>
</tr>
<tr>
<td>2nd radioactivity peak (Fig. 3.33)</td>
<td>1257</td>
<td>369</td>
</tr>
</tbody>
</table>
15 minutes than after 90 minutes of incorporation (Table 3.16). However, the washing procedure did not alter the distribution of radioactivity in the plasma membrane sheets isolated from the crude nuclear fraction. The washed membrane still showed more radioactivity after 90 minutes than after 15 minutes of the incorporation. Similar results were obtained after fractionation of the washed pellets and the salt extracts into perchloric acid-soluble and -insoluble fractions by the method of Sturgess et al. (1972). It was found that the radioactivity is mainly recovered in the perchloric acid-insoluble fraction as is the case on fractionation of rat serum into acid-soluble and -insoluble fractions (Table 3.17).

The material recovered in the microsomal subfractions (regions A, B, C, D and E, Fig. 3.33) was examined immunologically and compared with that of the plasma membrane sheets isolated from the crude nuclear fraction and the serum proteins separated by affinity chromatography on anti-(plasma membrane) antiserum conjugate. The pellets remaining after washing the membranes with 0.15 M-NaCl and 0.2 M-NaHCO₃ were extracted with 1% DOC + 0.5% Lubrol W (see section 2:6). An aliquot of the detergent extracts was allowed to diffuse against anti-(liver plasma membrane) and anti-(rat serum) antisera as described in section 2:11. The precipitation lines containing radioactively labelled glycoproteins were located by radioautography. The dry plates were left, in the dark, in contact with an X-ray film for about three months. The dry plates were later stained for proteins.
Table 3.16: Radioactivity recovered after washing the membrane fractions, from the short and long periods of fucose incorporation, with 0.15 M-NaCl and 0.2 M-NaHCO₃.

The results in the first and third columns are given as the radioactivity, measured in Lissapol-Scintillant, of the washed pellets recovered from 20 ml of pooled fractions shown in Fig. 3.32 and 3.33, whilst those in the second and fourth columns are the sums of the counts measured in the perchloric acid-soluble and insoluble fraction given in Table 3.17. The amount washed off as a percentage of the sums of radioactivity in the washed pellet and that in the salt extract is given in parentheses.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>15 min. after fucose injection</th>
<th>90 min. after fucose injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts/min in the washed pellet</td>
<td>Counts/min in the salt extracts</td>
</tr>
<tr>
<td>Regions from the B XIV rotor (Fig. 3.33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2295</td>
<td>4352 (65%)</td>
</tr>
<tr>
<td>B</td>
<td>3796</td>
<td>6016 (61%)</td>
</tr>
<tr>
<td>C</td>
<td>12753</td>
<td>11422 (47%)</td>
</tr>
<tr>
<td>D</td>
<td>13635</td>
<td>9184 (40%)</td>
</tr>
<tr>
<td>E</td>
<td>9222</td>
<td>6707 (42%)</td>
</tr>
<tr>
<td>Plasma membrane from the A XII rotor (region 4, Fig. 3.32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4634</td>
<td>1892 (29%)</td>
</tr>
</tbody>
</table>

* Some of the material of this region was lost during extraction with bicarbonate buffer. The radioactivity in the washed pellet and the bicarbonate extract was calculated according to the percentage removed from regions C and E.
Table 3-17: Radioactivity recovered, after 15 and 90 minutes of fucose incorporation, in the perchloric acid (PCA)-soluble and insoluble fraction isolated by the method of Sturgess et al. (1972) from 18 ml of the material extracted by 0.15 M-NaCl and 0.2 M-NaHCO₃, and from that in the washed membrane pellets recovered from 20 ml of pooled fraction. The results are presented as counts/min and (in parentheses) as a percentage of the sums of radioactivity recovered in all fractions. The overall recovery is given as a percentage of the total counts in the pooled fraction.

<table>
<thead>
<tr>
<th>Regions from the B XIV rotor (Fig.3-33)</th>
<th>SW* PCA-insoluble fraction</th>
<th>SW* PCA-soluble fraction</th>
<th>BW* PCA-insoluble fraction</th>
<th>BW* PCA-soluble fraction</th>
<th>WP* PCA-insoluble fraction</th>
<th>WP* PCA-soluble fraction</th>
<th>overall recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1205 (22.7%)</td>
<td>614 (11.58%)</td>
<td>1984 (37.4%)</td>
<td>548 (10.3%)</td>
<td>706 (13.3%)</td>
<td>245 (4.6%)</td>
<td>24%</td>
</tr>
<tr>
<td>B</td>
<td>1507 (16.1%)</td>
<td>570 (6.1%)</td>
<td>3165 (33.9%)</td>
<td>773 (8.3%)</td>
<td>3049 (32.7%)</td>
<td>244 (2.6%)</td>
<td>43%</td>
</tr>
<tr>
<td>C</td>
<td>3414 (13.4%)</td>
<td>1074 (4.2%)</td>
<td>5580 (21.9%)</td>
<td>1353 (5.3%)</td>
<td>13573 (53.4%)</td>
<td>404 (1.6%)</td>
<td>54.3%</td>
</tr>
<tr>
<td>D</td>
<td>2703 (10.4%)</td>
<td>793 (3.0%)</td>
<td>4571 (17.6%)</td>
<td>1117 (4.3%)</td>
<td>16370 (63.0%)</td>
<td>405 (1.56%)</td>
<td>56.8%</td>
</tr>
<tr>
<td>E</td>
<td>1972 (11.8%)</td>
<td>606 (3.6%)</td>
<td>3449 (20.6%)</td>
<td>859 (5.1%)</td>
<td>9570 (57.2%)</td>
<td>273 (1.6%)</td>
<td>45.7%</td>
</tr>
<tr>
<td>Plasma membrane from the A XII rotor (region 4, (Fig.3-32)</td>
<td>494 (8.17%)</td>
<td>302 (4.99%)</td>
<td>709 (11.7%)</td>
<td>386 (6.38%)</td>
<td>3912 (64.7%)</td>
<td>243 (4.0%)</td>
<td>48.2%</td>
</tr>
</tbody>
</table>
Table 3-17 contd.

Radioactivity in counts/minutes recovered in

<table>
<thead>
<tr>
<th></th>
<th>SW* PCA-insoluble fraction</th>
<th>SW* PCA-soluble fraction</th>
<th>BW* PCA-insoluble fraction</th>
<th>BW* PCA-soluble fraction</th>
<th>WP* PCA-insoluble fraction</th>
<th>WP* PCA-soluble fraction</th>
<th>overall recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>90 minutes of incorporation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regions from the B XIV rotor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>470 (15.6%)</td>
<td>271 (9.0%)</td>
<td>590 (19.6%)</td>
<td>271 (9.0%)</td>
<td>1221 (40.6%)</td>
<td>183 (6.0%)</td>
<td>54.9%</td>
</tr>
<tr>
<td>B</td>
<td>634 (10.59%)</td>
<td>335 (5.59%)</td>
<td>913 (15.25%)</td>
<td>308 (5.1%)</td>
<td>3612 (60.37%)</td>
<td>181 (3.0%)</td>
<td>73%</td>
</tr>
<tr>
<td>C</td>
<td>1371 (7.12%)</td>
<td>401 (2.08%)</td>
<td>1862 (9.67%)</td>
<td>428 (2.2%)</td>
<td>14942 (77.6%)</td>
<td>237 (1.23%)</td>
<td>75.9%</td>
</tr>
<tr>
<td>D</td>
<td>1386 (6.3%*)</td>
<td>463 (2.1%*)</td>
<td>2172 (9.9%*)</td>
<td>462 (2.1%*)</td>
<td>17176 (78.4%*)</td>
<td>239 (1.09%*)</td>
<td>2.2%**</td>
</tr>
<tr>
<td>E</td>
<td>1386 (5.66%)</td>
<td>441 (1.8%)</td>
<td>2482 (10.14%)</td>
<td>496 (2.02%)</td>
<td>19410 (79.3%)</td>
<td>243 (0.99%)</td>
<td>82%</td>
</tr>
<tr>
<td>Plasma membrane from the A XII run</td>
<td>709 (2.7%)</td>
<td>347 (1.3%)</td>
<td>1442 (5.5%)</td>
<td>814 (3.1%)</td>
<td>22298 (85.6%)</td>
<td>436 (1.67%)</td>
<td>99.26%</td>
</tr>
</tbody>
</table>

* Symbols used: SW, 0.15 M-NaCl extract; BW, 0.2 M-NaHCO₃ extract; WP, washed pellet recovered after washing the membrane successively with 0.15 M-NaCl and 0.2 M-NaHCO₃.

** The values have been corrected for the loss of material during the bicarbonate wash as described in Table 3-16.
No immunological activity was detected in the material of region A whereas the material of all other regions was indeed immunologically active against our anti-(plasma membrane) antiserum. A very weak reaction was detected between the material of region B and the anti-(plasma membrane). Fig. 3.34a and b shows that the protein pattern formed by the material of region C resembles that formed by the plasma membrane sheets more closely than does that of any of the other two regions. However, the precipitation lines formed by all three microsomal subfractions appear to be continuous with each other and with the corresponding lines formed by the plasma membrane sheets. This would suggest that the material in regions C, D and E is qualitatively but not quantitatively identical [the weak reaction against anti-(plasma membrane) of the material of region D after 90 minutes of fucose incorporation (Fig. 3.34b) could be attributed to the loss of material during extraction with bicarbonate buffer; this loss renders the final pellet more dilute; see Table 3.16 and 17]. Examination of Fig. 3.34c shows the presence of at least three precipitation lines formed by the material of region C. The major line formed appears to be continuous with one of the minor lines formed by the plasma membrane sheets. One of the weak lines, nearer to the antigen well, is common to the major line formed by serum against anti-(plasma membrane) antiserum and one of the major lines formed by the plasma membrane sheets. The other weak line, nearer to the antibody-containing well, which seems to be common to the three fractions, was found to be continuous with the major constituent of
The pattern of proteins on Ouchterlony double diffusion plates of 1% DOC +0.5% Lubrol W extracts of the pellets remaining after washing the membranes with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0, against anti-(liver plasma membrane) antiserum (A/PM) in the experiments involving: a) short, b) and c) long periods of fucose incorporation. Rat serum and the 2.5 M-MgCl₂ eluted serum fraction (ES) were included for comparison.

In all cases diffusion and precipitation were allowed to proceed for 48 hours at room temperature. The unprecipitated proteins were washed off with 2.5% NaCl and the dry plates were stained for proteins with Coomassie Brilliant blue R.
1) Serum
2) ES
3) Material from region C
4) Material from region D
5) Material from region E
6) Plasma membrane of region 4 from the A XII run (PM)
7) Material from region A
8) Material from region B
9) Anti-(plasma membrane) antiserum
the 2.5 M-MgCl₂ eluted serum fraction.

The radioautogram presented in Fig. 3.35a shows that after 15 minutes of fucose incorporation the material from region C produced the most radioactively labelled precipitation line against anti-(plasma membrane) antiserum. A very weakly labelled precipitation line was detected in the pattern formed by the material of the other fractions. After 90 minutes of fucose incorporation, it appears that the radioactivity in the precipitation lines formed by the material of region C and E was considerably enhanced (Fig. 3.35b). The very poor labelling of the precipitation lines formed by region D after 90 minutes of incorporation could be explained similar to that described for the weak protein precipitation lines (see Fig. 3.34b). The radioactively labelled precipitation line formed by the material of region E appears to be continuous with one of the minor lines formed by the plasma membrane sheets. Examination of Fig. 3.35c shows that the three precipitation lines formed by the material of region C shown in Fig. 3.34c were all radioactively labelled. The major line formed appears to be the most rapidly labelled whilst the other two weak lines are labelled only after 90 minutes of fucose incorporation. These results would suggest that the incorporation of fucose into liver plasma membrane takes place first in the membranes which are mainly recovered in the microsomal fraction after homogenisation and centrifugation. A high proportion of the rapidly labelled membrane glycoproteins appear to be concentrated in the low density microsomal vesicles which were also found to contain a
Radioautograms of the Ouchterlony plates shown in Fig 3-34. In all cases, the dry plates were left, in the dark, in contact with an X-ray film for about three months.
considerable amount of loosely bound material. Incorporation of fucose into the glycoproteins of the plasma membrane sheets isolated from the crude nuclear fraction appeared to be taking place at a relatively slow rate.

The immunological activity of the material in regions C, D, E and the plasma membrane sheets towards anti-(rat serum) antiserum was also examined as described earlier. When the dry plates were stained for proteins with Coomassie Brilliant blue R (Fig. 3.36a, b, c and d) no precipitation lines could be detected between any of the membrane fractions and anti-(rat serum). However, when radio-autograms of the same plates were produced (Fig. 3.37a, b, c and d) it showed that only after 15 minutes of fucose incorporation the material of regions C, D and E is capable of producing radioactively labelled precipitation line against anti-(rat serum). It is clear from Fig. 3.37b that the material of region C is more reactive towards anti-(rat serum) than any of the other fractions. However, after 90 minutes of fucose incorporation; no radioactively labelled precipitation line could be detected between any of the membrane fractions and the antiserum (Fig. 3.37c). This would indicate that the proteins that are common to rat serum and liver microsomal subfractions are only minor constituents of the microsomal subfractions. Nevertheless, it appears that those proteins contain the very fast and heavily labelled glycoproteins. Such common proteins are mainly concentrated in the material of region C which earlier in this chapter was demonstrated to be highly enriched with membrane vesicles mainly derived from the
Ouchterlony double diffusion plates of 1% DOC + 0.5% Lubrol W extracts of the pellets remaining after washing the membrane with 0.15 M-NaCl and 0.2 M-NaHCO₃. The diffusion was performed against anti-(rat serum) (A/S) and anti-(liver plasma membrane) (A/PM) antisera after short and long periods of fucose incorporation as indicated below. Rat serum (S) and the 2.5 M-MgCl₂ eluted serum fraction (ES) were included for comparison.

In all cases diffusion and precipitation were allowed to proceed for 48 hours at room temperature and the dry plates were stained for proteins with Coomassie Brilliant blue R.

The subscripts 1 and 2 represent the material after short and long periods of fucose incorporation respectively. The fractions on plate (c) correspond to those of plate (b) but are from the experiment with a long period of fucose incorporation. PM₁ and PM₂ are the material of region 4 from the A XII run (Fig. 3.32) after 15 and 90 minutes of incorporation respectively.
Radioautograms of the Ouchterlony plates shown in Fig 3-36. In all cases, the dry plates were left, in the dark, in contact with an X-ray film for about three months.
sinusoidal-facing area of the liver cell membrane. Hence it is likely that the glycoproteins present in region C that are common to rat serum are mainly secretory glycoproteins temporarily complexed with the membrane.
CHAPTER FOUR

DISCUSSION
DISCUSSION

The work described in this thesis was designed to elucidate whether the liver cell plasma membrane is the source, in normal rat serum, of enzymes such as alkaline phosphatase or 5'-nucleotidase which are elevated in human hepatic disease. The study was extended to examine the relationship between rat serum proteins in general and the hepatocyte plasma membrane. The major techniques used in this study were immunofluorescence and affinity chromatography. However, it is known that, in the liver cell plasma membrane there are two distinct, metabolically active, areas, the sinusoidal-surface and the biliary surface. As it was hoped to identify the part of the plasma membrane from which the proteins are released into the blood, methods had first to be developed to ascertain the subcellular source of the plasma membrane fragments which may be separated from the crude nuclear and the microsomal fraction. The results of these investigations will now be discussed.

Plasma membrane surface antigens and the properties of the anti-(plasma membrane) antiserum

The validity of the results presented in this thesis depends, to a considerable extent on the specificity of the anti-(plasma membrane) antiserum. To prepare the latter required the isolation of purified plasma membrane for use as an immunogen. Rat liver plasma membranes were isolated by the method of Prospero and Hinton (1973). Earlier studies by Emmelot et al. (1964) showed that the isolated membranes
contain proteins that are soluble in physiological saline. Among those saline-soluble components are proteins common to rat serum which may be absorbed onto the membrane after homogenisation. In addition non-membraneous proteins may be trapped inside the membrane vesicles. These trapped proteins are reported to be removed by washing the membrane with 0.2 M-bicarbonate buffer, pH 9.0 (Weihing et al., 1972).

Hence, in the present investigation the membrane preparations used to raise the antiserum were extracted with 0.15 M-NaCl and 0.2 M-bicarbonate buffer, pH 9.0 to remove non-membraneous proteins.

The presence, in our anti-(plasma membrane) antiserum, of antibodies against a number of enzymic activities that are predominantly located in the plasma membrane was demonstrated by histochemical staining (Fig. 3-7). Fluorescein-labelled anti-(plasma membrane) was also used to study the binding of the antiserum to the isolated plasma membrane. The intra-membrane location of the antigens was studied by examining the effect of the washing procedures on the binding of the antibodies to the membrane. As demonstrated in the Results section, washing the isolated plasma membrane with 0.15 M-NaCl and 0.2 M-bicarbonate buffer caused a considerable increase in the extent of binding of the fluorescein-labelled anti-(plasma membrane) to the washed membrane. This indicates that the washing procedure results in the exposure of new antigenic binding determinants on the membrane surface which were previously masked. Masked membrane sites were demonstrated by Gurd et al. (1972) in mouse liver plasma membrane by treating the membrane with 50 mM-bicarbonate
buffer. Exposure of previously masked antigenic sites of rat liver plasma membrane was reported by Sheffield and Emmelot (1972) after treating the membrane with papain. The masking phenomenon has been reported also in other context, e.g. in transformed tumour cells (Hayry and Defendi, 1970) and in the untransformed membrane after treatment with trypsin (Makita and Sayama, 1971). Un-masking of hormone receptors in the liver has been demonstrated by studying the effects of treatment of the membrane with hydrolytic enzymes (Cuatrecasas, 1971). These observations are consistent with the increased binding of anti-(plasma membrane) antiserum to our salt-washed membrane as compared with the unwashed membranes.

Antigenic groups common to rat serum and liver plasma membrane

The relationship between liver plasma membrane and rat serum was first studied by examining the binding of fluorescein-labelled anti-(rat serum) to the isolated plasma membrane. This showed that about 1% of the protein in the globulin fraction of the anti-(rat serum), as against 0.16% of globulin from non-immunized rabbit, was capable of binding to the isolated plasma membrane. After washing the membrane with 0.15 M-NaCl and 0.2 M-NaHCO$_3$ buffer, it appears that the binding capacity of the washed membrane was decreased considerably. The proportion of anti-(rat serum)-binding material removed during washing is about the same as the proportion of the total membrane proteins which is solubilized (Table 3-5). Thus it was concluded that the membrane antigenic groups that are common to rat serum must be largely
exposed on the membrane surface and not masked by the loosely bound proteins removed during the washing procedure as are the true membrane proteins which bind the anti-(plasma membrane) antiserum.

As a considerable proportion of the membrane proteins capable of binding to anti-(rat serum) were removed in the washing procedure, it was decided to examine the solubilized proteins more closely. Examination of the immunological reaction of the salt extracts with anti-(cell cytosol), anti-(rat serum) and anti-(liver plasma membrane) antisera showed the presence of proteins which can react with the three antisera. Absorbed cytosol proteins were mainly recovered in the 0.15 M-NaCl wash, whereas materials homologous to rat serum proteins were mainly extracted by the bicarbonate wash. Material reacting with anti-(plasma membrane) antiserum but not cross-reacting with anti-(rat serum) or anti-(cell cytosol) was also detected in both washes. This confirms that at least part of the loosely bound proteins are true membrane proteins which mask binding sites located deeper in the membrane. Six plasma membrane proteins were detected in the salt extract, two of which were found to be common to rat serum. However, although washing the isolated plasma membrane caused the removal of some of the proteins common to rat serum and liver plasma membrane, enough of those proteins are firmly associated with the membrane and are present in sufficient amount to produce antibodies when the washed membranes were used as an immunogen. As the washing procedure should remove proteins absorbed from the serum, these antigenic components are presumably proteins released in vivo.
from the liver cell membrane into the blood.

The postulate presented above was supported by the observation that our anti-(plasma membrane) antiserum is capable of producing immunoprecipitation lines when tested against rat serum (Fig. 3-16a). Therefore, it is clear that the common proteins could be isolated if the proper technique was used. Affinity chromatography provides a quick and specific method for isolating minor components from a mixture of proteins (Cromwell, 1973). Accordingly, affinity chromatography of rat serum on immobilised anti-(plasma membrane) was decided to be the approach to isolate serum proteins common to rat liver plasma membrane. In the trial experiments, advantage was taken of the availability of anti-(cell cytosol) antiserum. This was used, with cell cytosol, as a model for specific antigen-antibody reaction, to verify the affinity chromatography conditions. From these trial experiments it was concluded that the antibodies covalently bound to the Sepharose 4B retain their capacity to bind the corresponding antigens and the coupled antigens could be detached by a method which did not destroy their enzyme activity.

Proteins common to rat serum and liver plasma membrane were isolated by affinity chromatography of serum on immobilised anti-(plasma membrane). Under our experimental conditions about 4% of rat serum proteins reacted with the anti-(plasma membrane) conjugate. Enzyme measurements on the unbound material and on fractions eluted from the column (ES) showed that almost none of the 5'-nucleotidase or
alkaline p-nitrophenyl phosphatase was bound to the immobilised antibodies, in spite of these enzymes being, in the liver, predominantly associated with the plasma membrane. There are four possible explanations for these results. It is possible that initially no antibodies to those enzymes were raised in our anti-(plasma membrane) preparation, or the antibodies were destroyed during the coupling procedure, or they are not covalently bound to the activated Sepharose. Alternatively, liver plasma membrane is not a major source of these enzymes in serum.

The first explanation appears unlikely because of the following findings. In the trial experiments with liver cell cytosol and anti-(cell cytosol) antiserum, about 88% of the 5'-nucleotidase and 87% of the alkaline p-nitrophenyl phosphatase activities were bound to the immobilised antiserum. This shows that the antibodies to these enzymes are indeed coupled to the activated Sepharose and retain their biological activities. However, due to the fact that the soluble nucleotidase is different from that associated with particles (Tjernshaugen and Fritzson, 1976) one cannot be certain that the antibodies to plasma membrane 5'-nucleotidase in our anti-(plasma membrane) did bind to the Sepharose. The results obtained from the trial experiments did, however, show that it is possible to couple the anti-(enzyme) antibodies to the activated Sepharose.

Histochemical staining of the immunoprecipitation lines formed by solubilized plasma membrane against anti-(plasma membrane) showed
the presence of antibodies to most of the plasma membrane enzymes (Fig. 3.7), in spite of the inhibition effect of the antibodies on the enzyme activity (Evans et al., 1973a). 5'-Nucleotidase activity was found in two lines with the same electrophoretic mobilities, besides there were three lines each of which contain alkaline p-nitrophenylphosphatase, L-leucyl-β-naphthylamidase (LNase) and ATPase activities. This may suggest the presence, in the liver cell plasma membrane, of a multienzyme complex. A similar complex exhibiting LNase, nucleoside di- and triphosphatase activities has been reported by Blomberg and Raftell (1974) using two dimensional immunoelectrophoresis. Although these authors did not assay for 5'-nucleotidase, Blomberg and Perlmann (1971b) reported that the activity of 5'-nucleotidase was present in two precipitation lines, different from those in which ATPase activity was detected. Similar results were observed by Simon and Thomas (1972) on polyacrylamide gel electrophoresis of homogenates of the membrane of the pancreas. They reported that 5'-nucleotidase is not associated with the proteins which contain LNase and alkaline p-nitrophenylphosphatase activities. They interpret the latter as indicating the presence of a multienzyme complex on the plasma membrane. Our histochemical results support the presence of a multienzyme complex on the liver cell plasma membrane and suggest that 5'-nucleotidase is probably not associated with this complex. This difference in the behaviour of 5'-nucleotidase from other plasma membrane enzymes was also observed when the effect of deoxycholate + Lubrol W on the plasma membrane enzymes was studied. 5'-Nucleotidase activity
was found to be enhanced up to 300%, whereas LNase and alkaline
p-nitrophenyl phosphatase activities were inhibited by about 45%
and 30% respectively. An inhibition by the same detergent of the
activity of plasma membrane ATPase appeared in the report of
Blomberg and Perlman (1971b) in which they also report that the
detergents enhance 5'-nucleotidase. To conclude, both the results
of the studies on the effect of the detergent on the four enzymes and
the histochemical results may be attributed as due to differences in
location, within the membrane, of 5'-nucleotidase and the other
enzymes mentioned above. From these results and those from other
experiments, as discussed below, it seems most likely that antibodies
against "plasma membrane enzymes" are present in our anti-(plasma
membrane) preparation. As there seems no good reason why they
should not be covalently bound to the activated Sepharose or should
be selectively inactivated, it was concluded that the hepatocyte
plasma membrane is not a major source of the 5'-nucleotidase and
alkaline phosphatase in normal rat serum.

This postulate was supported by the results obtained by colleagues
at the Centre during preparation of this thesis. When serum from rats
made jaundiced by ligation of the bile duct for three days was
fractionated by affinity chromatography on immobilised anti-(plasma
membrane), only about 17% of the 5'-nucleotidase activity was recovered
in the unbound serum protein fraction, although only 7% could be
recovered in the fractions eluted by 2.5 M-MgCl₂ (Issa et al., 1976).
The low recovery of the enzyme activity is probably due to denaturation
of this enzyme by the eluting agent. Those observations showed that active antibodies to 5'-nucleotidase were present on the column and so confirmed that, although the additional 5'-nucleotidase which appears in serum of jaundiced rat is derived from the liver plasma membrane, the activity present in normal serum comes from a different source. These results are consistent with the reports of Righetti and Kaplan (1971) that alkaline phosphatase in normal rat serum is of bone origin and it is also in line with that given by Kryszewski et al. (1973) that 5'-nucleotidase in serum of rats made jaundiced by ligation of the common bile duct is of hepatic origin.

Serum alkaline phosphatase, however, appears to behave in a way different from that of 5'-nucleotidase after ligation of the common bile duct. Measurement of serum alkaline phosphatase showed a considerable increase in the activity of this enzyme when assayed with β-glycerophosphate as substrate, whereas there was very little increase in the activity when p-nitrophenylphosphate was used. When serum of jaundiced rats was fractionated by affinity chromatography on immobilised anti-(plasma membrane), alkaline phosphatase activity (with β-glycerophosphate or p-nitrophenylphosphate as substrate) was mainly recovered in the unbound serum protein fraction. These results support our previous conclusion that the plasma membrane of normal liver is not a major source of alkaline phosphatase in serum of jaundiced rats. The results obtained from fractionation of serum of jaundiced rats were in accordance with expectation, since plasma membrane isolated from normal rat liver showed only alkaline p-nitro-
phenylphosphatase activity but no alkaline β-glycerophosphatase activity (Emmelot et al., 1964). Hence one would expect that serum alkaline β-glycerophosphatase would not bind to the immobilised anti-(plasma membrane). However, the hepatic origin of the additional activity of serum alkaline β-glycerophosphatase could not be excluded. Emmelot et al. (1964) reported that a remarkable increase occurred in alkaline β-glycerophosphatase activity in the plasma membrane isolated from rat liver after ligation of the common bile duct. This was attributed as due to the presence of a new enzyme different from that hydrolysing p-nitrophenylphosphate.

A possible interpretation of the results of Emmelot et al. (1964) is that the increased level of alkaline β-glycerophosphatase activity in the plasma membrane is due to the de novo synthesis of this enzyme by the liver reported by Kaplan and Righetti (1970) and Kryszewski et al. (1973). Consequently the suggestion can be made that alkaline β-glycerophosphatase and p-nitrophenylphosphatase in rat serum are two different enzymes. At present there is insufficient evidence to support this suggestion, but our results suggest that alkaline p-nitrophenylphosphatase and β-glycerophosphatase are behaving differently during obstructive jaundice. Hence, the sensitivity of serum alkaline phosphatase and consequently its clinical reliability should be considered with its substrate specificity (see Introduction, p.12). It is very difficult to extrapolate our experimental results on normal rats and on rats suffering from acute biliary obstruction to diseases in humans which might be chronic and probably due to
several factors. However, it seems that our findings and that of others are in line with the clinical observations on humans (Phelan et al., 1971) as outlined in the 'Introduction'.

When normal rat serum was fractionated by affinity chromatography, L-leucyl-β-naphthylamidase and alkaline RNase (assayed at pH 7.8 in the presence of 50 mM-MgCl₂), activities were detected in the fractions recovered by eluting with 2.5 M-MgCl₂. From these assays it appears that about 8% of the L-leucyl-β-naphthylamidase and 10% of the RNase activities in serum is associated with the proteins common to rat serum and liver plasma membrane. Supporting evidence for this biochemical observation is derived from the results of polyacrylamide gel electrophoresis of the material common to rat serum and liver plasma membrane. L-Leucyl-β-naphthylamidase activity was found by histochemical staining to be associated with proteins migrating to the α₁ position. These results are consistent with those of Smith and Rutenburg (1963) who found that, on electrophoresis on cellulose acetate strips, both serum and liver aminopeptidase migrate in the α₁ position. Similarities between liver and serum aminopeptidase has been reported by Behal et al. (1966). Hence it was concluded that the hepatocyte plasma membrane is almost certainly the source of part of the serum L-leucyl-β-naphthylamidase activity and probably of serum RNase. However proteins with enzyme activity do probably form only a small proportion of the serum proteins which bind to immobilised anti-(plasma membrane) antiserum, so it is of interest to know what are the other common
proteins and if possible how such materials are released from the plasma membrane into the blood.

Proteins common to rat serum and liver plasma membrane

Immunoelectrophoresis against anti-(plasma membrane) and anti-(rat serum) antisera of the material bound to the immobilised anti-(plasma membrane) and eluted by 2.5 M-MgCl₂ (ES) showed the presence of at least seven precipitation lines which were identical for the two antisera (Fig. 3.22a). Three of the common lines were associated with proteins migrating in the β-region, two lines with proteins which remain at the origin, and one line each with proteins migrating in the γ-globulin and albumin regions. A few other lines were only detected against anti-(rat serum).

Gradient polyacrylamide gel electrophoresis confirms the presence of albumin in the "ES" fraction (Fig. 3.20). This was not unexpected since it is known that this protein is synthesised in, and secreted from, the liver cell (Peters, 1962; Glauman, 1970). The presence in the "ES" fraction of proteins that migrate in the γ-globulin region was unexpected, but this protein was also detected, although to a less extent, in the material extracted by 0.15 M-NaCl and 0.2 M-NaHCO₃ buffer (Fig. 3.11), and may be due to the binding of especially basic protein to the acidic membrane surface.

Electrophoresis of a concentrated sample of "ES" fraction on polyacrylamide gel gave eight bands when the gel was stained for
proteins (Fig. 3.21a), this agreed with the number of proteins obtained by immunoelectrophoresis. Three bands, one migrating to the $\alpha_1$ and two to the $\beta$-globulin regions, were positively identified as glycoproteins by staining the gel for carbohydrate with periodic acid-Schiff reagent. This would indicate that those two types of serum glycoproteins originate from liver plasma membrane. This suggestion was supported by results from experiments on labelling serum glycoproteins with $^{14}\text{C}]fucose$. Fig. 3.31 shows that the proteins common to rat serum and liver plasma membrane that migrate in the $\beta$-globulin region are almost certainly glycoproteins. However, there was no positive evidence for the proteins migrating to the $\alpha_1$ position as being glycoproteins.

Comparison of the pattern, on immunoelectrophoresis, against anti-(plasma membrane) formed by rat serum with the pattern formed by (ES) fraction showed that almost all the common proteins were successfully recovered from the affinity column (Fig. 3.22). The only missing protein from the "ES" fraction is that responsible for the formation of line 1. This could be explained by either of the two possibilities. First, the antibodies against this particular protein are not covalently bound to the activated Sepharose under our experimental conditions. Alternatively, there is some sort of degradation of this type of serum protein due to the eluting agent. Unfortunately, we could not test either of the two possibilities, but it appears that the missing protein is a glycoprotein which is strongly
labelled with fucose after only 90 minutes of incorporation (Fig. 3.31). Thus, it seems that this glycoprotein is different from the other glycoproteins. It is very possible that breakdown of this serum protein into subunits is taking place due to the low pH of the eluting agent.

Therefore, it is clear that there are proteins common to rat serum and liver plasma membrane. Among those common proteins, carbohydrate-rich proteins were found to be mainly of the \( \beta \)-globulin type. Those common components could be either transient components temporarily complexed with the membrane, or part of the structural protein of the membrane. As the liver cell plasma membrane is not a uniform structure but contains an area facing the sinusoid which is differing functionally and morphologically from the area facing the bile canaliculi, it is of clinical importance to understand how and from which part of the plasma membrane such proteins are released.
As described earlier, in the liver cell plasma membrane there are two distinct metabolically active areas: the sinusoidal-surface and the biliary surface. As it was hoped to identify the part of the plasma membrane from which the proteins are released into the blood, these two areas had to be isolated and characterised. It has been suggested, though without definite proof, that the plasma membrane vesicles recovered in the microsomal fraction are derived largely from the sinusoidal-surface of the liver cell (Hinton et al., 1971). Hence an attempt was made to separate those vesicles from the bulk of the microsomes (i.e. the endoplasmic reticulum fragments) by an isopycnic flotation of rat liver microsomes in a linear sucrose density gradient. The distribution, through the gradient, of a number of the most commonly used plasma membrane marker enzymes was studied. The results of these experiments showed that flotation of a microsomal suspension yields a zone at a density of 1.14 g/ml which is enriched in 5'-nucleotidase (Fig. 3.25). The proportion of 5'-nucleotidase activity found in this region was found to be comparable to the amount found in "microsomal plasma membrane" as separated by Touster et al. (1970) using a step gradient. However, this low density fraction is not typical of all plasma membrane fragments of microsomal size for, as shown in the 'Results' section, other plasma membrane enzymes, namely alkaline p-nitrophenyl phosphatase, alkaline phosphodiesterase, L-leucyl-β-naphthylamidase (LNase),
ADPase and ATPase activities, form a band centred at a density of about 1.165 g/ml. The particles which form this band almost certainly contain 5'-nucleotidase, for a distinct shoulder of activities was apparent at a density of 1.165 g/ml. These results demonstrate that 5'-nucleotidase does not have the same distribution among the population of the vesicles present in the microsomal fraction as that of other enzymes which are supposedly located in the plasma membrane. There are three possible explanations of these results:

a) There may be variation in the accessibility of the enzymes to the substrate.

b) Either 5'-nucleotidase or the other enzymes may be partly located in cell structures, other than the plasma membrane, that form vesicles of microsomal size on tissue disruption.

c) The plasma membrane fragments of microsomal size may be enzymically heterogeneous.

El-Aaser et al. (1973) reported cytochemical evidence which indicates that 5'-nucleotidase is located on the outer face of the plasma membrane vesicles separated from microsomal fraction. If the other enzymes were inside the vesicles and the denser vesicles were either more easily penetrated by the substrate or the vesicularization were "inside out" (Wallach, 1967), then our results would be explained. However, if permeability played any role, then addition of detergent to the enzyme assay mixture should produce equalisation of the enzyme
distribution patterns. As the addition of 1% Triton X-100 to the incubation mixture did not affect the enzyme distribution pattern, our results could not be explained by variation in the accessibility of the enzyme to the substrate. The second way in which our results could be explained is that 5'-nucleotidase or the other enzymes are partly located in cell structures other than the plasma membrane, which possibly contaminate our microsomal fraction. This possibility will now be discussed in more detail.

Some 5'-nucleotidase activity has been demonstrated in the Golgi apparatus (Bergeron et al., 1973), from which our low density vesicles may be derived. However, the results of Fleischer and Fleischer (1969, 1970) showed that the ADPase activity present in the Golgi apparatus (Cheetham et al., 1970) bands at a density much lower than that of the plasma membrane fragments in which most of the 5'-nucleotidase is recovered. If our preparation had contained a significant amount of Golgi apparatus, a peak of ADPase should have been found at a density less than 1.14 g/ml. However, no such peak was detected.

From work on lysosomes (Dobrota and Hinton, 1974) it is clear that acid β-glycerophosphatase is associated with lysosomal membrane banding at a density of 1.165 g/ml. The results of our microsomal flotation experiments showed that the acid β-glycerophosphatase peak lies at the same density as that reported for the lysosomal fragments (M. Dobrota, unpublished results). Even if all this acid phosphatase activity is attributable to contaminating lysosomal fragment which might appear in our
microsomal fraction, a comparison with the results for disrupted lysosomes (M. Dobrota, unpublished results) showed that only a very small proportion of the "plasma membrane enzymes" in this region can be attributed to the presence of lysosomal membranes. Mitochondrial inner membranes are another possible source of the low density vesicles, since ATPase is present in mitochondria (Sabatini et al., 1964). However measurement of succinate dehydrogenase activity showed that negligible proportion of this subcellular component was recovered in the microsomal fraction. The contribution of mitochondrial outer membrane to our results has not been examined during this investigation, but observations by colleagues at the Centre (Hinton et al., 1971; Norris, 1973) showed that the extent of contamination is very low as judged by the activity of monoamine oxidase. Our results also indicated that the 5'-nucleotidase reported in the rough endoplasmic reticulum (Widnell, 1972) can be responsible for no more than 10% of the 5'-nucleotidase of the microsomal fraction.

Cytochemical evidence (Goldfischer et al., 1964) and cell-fractionation experiments (Emmelot et al., 1964; Wattiaux-deConinck and Wattiaux, 1969; Thines-Sempoux, 1973) indicate that 5'-nucleotidase, ADPase, ATPase and alkaline p-nitrophenyl phosphatase are all located mainly in the plasma membrane. Prospero et al. (1973) suggested that magnesium-activated alkaline phosphodiesterase is also restricted to the liver cell plasma membrane. The latter enzyme and 5'-nucleotidase have, in fact, been used as markers for plasma membrane fragments isolated from the microsomal fraction (Touster et al.,
1970). This evidence, together with our histochemical results (Fig. 3.26), indicates that the microsomal subfractions that contain 5'-nucleotidase consist entirely of plasma membrane fragments, but that the fragments do not contain a uniform complement of enzymes.

This conclusion is strengthened because a very similar heterogeneity was found when the plasma membrane of density 1.17 g/ml isolated from the crude nuclear fraction was disrupted. Two subfractions of densities 1.12 and 1.18 g/ml were obtained (Evans et al., 1970a). These two subfractions were reported to be morphologically and enzymatically different. The vesicles of the light subfraction were more highly enriched in 5'-nucleotidase than the sheets of the denser fraction. Isolation of such a light subfraction was also reported by Thines-Sempoux (1973). The latter author was able to detect a group of vesicles enriched in 5'-nucleotidase with a low median density as compared to that of the smooth and rough endoplasmic reticulum.

Although the distribution pattern of monoamine oxidase, a marker for the outer mitochondrial membrane, was reported to be similar to that of 5'-nucleotidase, these two enzymes could be separated after digitonin treatment (Amar-Costesc et al., 1974).

Insulin and glucagon are found in bile at a concentration similar to that in the portal vein (Buchanan et al., 1968), and adenyl cyclase has been shown to reside in at least two different morphological sites in the hepatic tissue, the bile canalicular and the sinusoidal-surfaces (Reik et al., 1970). The binding sites of these hormones have been
used in reported studies of the distribution of the sinusoidal surface after homogenisation of the liver. House and Weidemann (1970) reported that light vesicles isolated from the microsomal fraction, by centrifugation in a discontinuous Ficoll gradient, were highly enriched in insulin-binding sites as compared with two plasma-membrane subfractions of densities 1.17 and 1.18 g/ml isolated from the crude nuclear fraction by a method comparable to that of Touster et al. (1970). Hence, they suggested that the light microsomal vesicles, which band in Ficoll at a density of 1.036 g/ml, are derived largely from the sinusoidal-surface of the hepatic cell. Apparently contrary results emerged from studies by Evans et al. (1973b) in which an insulin-binding activity was demonstrated in the vesicles of densities 1.16 and 1.18 g/ml isolated by vigorous rehomogenisation of the plasma membrane isolated from the crude nuclear fraction. At that time, these authors concluded that these two subfractions are enriched in vesicular elements of the hepatocyte surface derived from the sinusoidal face and the intracellular face. Their results confirm the absence of the hormone-binding activity in the subfraction of density 1.13 g/ml separated from the crude nuclear fraction.

The apparently conflicting results mentioned in the last paragraph have recently been reconciled by Wisher and Evans (1975) who investigated, in some detail, the subcellular origin of the three subfractions of densities 1.13, 1.16 and 1.18 g/ml obtained from the plasma membrane isolated from the crude nuclear fraction and in the microsomal subfraction of density 1.13 g/ml isolated by the method of
Touster et al. (1970). Glucagon-sensitive adenyl cyclase was used as a marker enzyme for the sinusoidal fragments. They reported that the highest degree of glucagon-stimulated adenyl cyclase activity was found in the microsomal subfraction of density of 1.13 g/ml. Hence, they concluded that this fraction is derived largely from the sinusoidal-surface of the liver cell. Although considerable adenyl cyclase was still detected in subfractions from the plasma membrane sheets separated from the crude nuclear fraction, this enzyme was not stimulated by glucagon. Hence, it was suggested that the subfraction of density 1.13 g/ml isolated from the plasma membrane sheets of the crude nuclear fraction is composed of a mixture of smooth vesicles derived largely from the bile canalicular surface. The properties of the microsomal subfraction of density 1.13 g/ml studied by Wisher and Evans (1975) resemble those of our low density microsomal subfraction, which suggests that the latter is probably derived largely from the sinusoidal surface.

In the experiments described in this thesis a more direct method was used to determine the cellular origin of the subfraction obtained by flotation of the microsomal fraction. An apparently successful labelling of the sinusoidal surface was achieved using 4-acetamido-4'-iso-thiocyanato-stilbene-2,2'-disulphonic acid disodium salt (SITS) (Fig. 3.27). However, practical problems arose when it was attempted to use the bound SITS as a marker for the sinusoidal fragments in the subcellular fractionation experiments. The problems associated with measuring the fluorescence of the fractions recovered from
fractionation of the crude nuclear fraction and the microsomal fraction obtained from livers perfused with SITS, discussed in the "Results" section, did not permit a definite localisation of the sinusoidal surface among the fractions. However, a tentative placement may be possible. In agreement with the results of Wisher and Evans (1975), the sinusoidal surface of the hepatic cell appears to be the source of the low-density vesicles isolated from the microsomal fraction. A much smaller proportion of the sinusoidal surface membranes appears to be present in the plasma membrane sheets isolated from the crude nuclear fraction. This conclusion is strengthened because of the results obtained from experiments on the incorporation of $^{14}$C fucose into glycoprotein fractions of liver plasma membrane discussed below.
Incorporation of L-$^{14}$C fucose into serum and liver plasma membrane glycoproteins

Two mechanisms have been postulated by which material can reach the liver cell membrane for secretion. First, the synthesised protein, like "export" proteins in tissues such as the pancreas, may pass through the endoplasmic reticulum to the Golgi apparatus then to the plasma membrane for secretion. The second suggested pathway is that the proteins pass directly from the endoplasmic reticulum directly to the plasma membrane (Riordan et al., 1974; Frank et al., 1971). Whatever the pathway, protein reaching the extracellular part of the plasma membrane for secretion may either be released immediately or temporarily form part of the membrane coat and then be released into the bloodstream. Serum and membrane glycoproteins labelled after a very short pulse of radioactive fucose were examined in an attempt to differentiate the two hypotheses. As the proteins common to liver plasma membrane and rat serum, isolated by affinity chromatography, contain at least some glycoproteins, L-$^{14}$C fucose was used as a precursor of glycoprotein to investigate the release of glycoproteins from the liver cell plasma membrane.

The results obtained by affinity chromatography of rat serum on immobilised anti-(plasma membrane) conjugate showed that after a short period of fucose incorporation the percentage of radioactively labelled serum proteins that are capable of binding to our column is
higher than that after a long period of incorporation. These results indicate that serum proteins that are common to rat liver plasma membrane are especially rapidly labelled with $L^{14}\text{C}$ fucose. This would suggest that the proteins reaching the extracellular part of the plasma membrane for secretion must be especially rapidly labelled with $[^{14}\text{C}]$ fucose. However, these proteins must be, at least temporarily, complexed with the membrane during secretion in such a way that this association persists after isolating and washing the membrane before it is used as an immunogen. Supporting evidence for this conclusion comes from the results of Riordan et al. (1974) who found that the portion of the deoxycholate-solubilized plasma membrane, isolated by their method, that was precipitable with anti-(rat serum) antiserum showed the highest radioactivity 15 minutes after intravenous injection of $L-[^{14}\text{C}]$ fucose. This amount of radioactivity had decreased markedly after 30 minutes. However, there was a slight further fall between 30 and 60 minutes of incorporation, although the incorporation in the Golgi apparatus showed the expected exponential fall of radioactivity. This suggested that some of the proteins common to liver plasma membrane and rat serum are of the slowly labelled, non-secretory class. This observation is not discussed by these authors, but it seems reasonable and compatible with the results presented in this thesis.

Thus, it seems reasonable to suggest that the rapidly labelled membrane glycoproteins include at least a proportion of the secretory glycoproteins. As the microvilli of the blood-sinusoidal face of the
hepatocyte are the elements specialised for exchange of metabolites with the blood, it seemed likely that the radioactively labelled glycoproteins would be concentrated in the sinusoidal membrane fragments in experiments such as those described in section 2.5 a and b.

As demonstrated in the Results section, after labelling for either 15 or 90 minutes with fucose a considerably greater proportion of radioactivity was found in the microsomal subfractions than in the plasma membrane sheets isolated from the crude nuclear fraction. This would indicate that the incorporation of fucose into liver plasma membrane glycoproteins takes place first in membranes which are mainly recovered in the microsomal fraction after homogenisation and centrifugation. A high proportion of the rapidly labelled glycoproteins appeared to be concentrated in the material banding at a density of 1.14 g/ml (Fig. 3.33), which was found to contain a considerable amount of loosely bound material (Table 3.16). As this fraction produced the highest radioactively labelled precipitation lines when tested against anti-(rat serum), it seems that the secretory glycoproteins are mainly concentrated in this microsomal subfraction. Moreover, the secretory glycoproteins appear to be, at least temporarily, complexed with the membrane in such a way as to survive the isolation and the washing procedure.

For the reasons described above it was concluded that fragments from the sinusoidal surface of the liver cell plasma membrane are mainly concentrated in the microsomal subfraction of density 1.14 g/ml.
This supports the conclusion drawn from the experiments on in situ labelling of this surface with SITS. The low fluorescence found in the plasma membrane sheets may also be attributed to the presence of vesicles probably derived from the sinusoidal surface, as in the same fraction there is a relatively low radioactivity after 15 minutes of fucose incorporation. However, after 90 minutes of fucose incorporation the radioactivity, like most "plasma membrane" enzymes, was mainly concentrated in the microsomal subfraction of density 1.16 g/ml. This change in the radioactivity pattern was accompanied by a substantial increase in the radioactivity of the plasma membrane sheets. These shifts may be explained by movements of molecules within the membrane, as discussed below.

In the liver, the bile canaliculi are connected to the blood-sinusoidal surface by a flat area of membrane which contains the junctional complexes which link the neighbouring cells. If the labelled glycoproteins move from the sinusoidal surface to the contiguous part of the plasma membrane by way of flow within the membrane (Wallach, 1972), then fragments of the membrane of the contiguous face could well be concentrated in the microsomal subfraction of density 1.165 g/ml. A similar hypothesis has been put forward for the movement of material within the endoplasmic reticulum membrane (de Pierre and Dallner, 1975).

The hypothesis that the band centred at a density of 1.165 g/ml derives from the contiguous membranes was strengthened by the results
of the immunological examination of the microsomal subfractions against anti-(rat serum) antiserum. This showed that the radioactivity in the precipitation lines decreased as the density of the fraction increased, which indicates that the contiguous membranes, which supposedly band at a density of 1.165 g/ml, are also accessible to the circulating blood, confirming the results of Goodenough and Revel (1970). This would provide an explanation for the distribution of bound SITS among the fractions of the B XIV run, for fluorescence was spread over both the low density vesicles and those banding at about 1.165. However, the results of Wisher and Evans (1975) showed that part of the contiguous areas of the hepatocyte remain attached to the large sheets of membrane which sediment with the crude nuclear fraction.

General comments

Correct diagnosis of liver disease depends very largely on the estimation of certain enzymes in serum. Among those enzymes, 5'-nucleotidase and alkaline phosphatase are largely located in the plasma membrane of hepatocytes. These enzymes were however shown, by the experiments described in this thesis, not to be among the proteins common to normal rat serum and liver plasma membrane. However, in obstructive jaundice the additional 5'-nucleotidase in serum was found to be almost certainly derived from the hepatocyte plasma membrane, whereas the additional alkaline phosphatase in serum appears not to be the same as that found in the hepatocyte membrane of normal rat.
These results accord with the finding that the rise in serum alkaline phosphatase in obstructive jaundice is intimately related to the de novo synthesis of this enzyme by the liver (Kaplan and Righetti, 1970) whereas the additional 5'-nucleotidase in jaundiced rat serum showed a different response to feeding drugs, such as cycloheximide, that are known to inhibit protein synthesis in the liver (Krysiewski et al., 1973). Whatever the mechanism by which serum alkaline phosphatase is elevated, it seems that the distinctive alkaline phosphatase activity which appears in the hepatocyte plasma membrane after ligation of the common bile duct (Emmelot et al., 1964) is a possible source of the additional alkaline phosphatase in jaundiced rat serum. However, in normal or jaundiced rats, two possible ways may be suggested by which the material may enter the blood; a direct release from the sinusoidal surface of hepatic or sinusoid-lining cells, or a release from the biliary surface of hepatic or bile duct epithelial cells. It is therefore clear that the differential diagnosis of liver disease would be helped if it were known how the material is released and from which part of the plasma membrane.

Examination of serum proteins after injection of L-\(^{14}\)C] fucose showed that the proteins common to liver plasma membrane and rat serum are among the rapidly labelled secretory proteins. In addition, plasma membrane fragments from the areas of the liver cell facing the sinusoid seem to be labelled more rapidly than other plasma membrane fragments. The shift of radioactivity, after a long period of incorporation, from the material banding at a density of 1.14 g/ml to that banding at 1.165 g/ml,
and the substantial increase in radioactivity in the plasma membrane of the crude nuclear fraction, may indicate the presence of a unidirectional flow of membrane from the sinusoid to the other surfaces of the liver cell.

These results, taken together, suggest that the plasma membrane of liver cells may play a complex role in the release of material to the blood. The nature of this role could be examined by study of the rate of labelling of selected proteins in different membrane subfractions. Membrane proteins can be subfractionated by polyacrylamide gel electrophoresis in the presence of a suitable detergent, and the distribution of labelled glycoprotein can be determined. A comparison of the glycoprotein labelling pattern of the membrane subfraction with that of serum proteins including those homologous to plasma membrane may indicate whether there is a significant "washing off" of proteins on the cell surface in addition to the discharge of proteins in secretion granules. This also may help to determine whether the enzymes appearing in the serum of jaundiced rats derive from the sinusoidal or the bile canalicular face of the liver cells.
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