HEPATOMA PLASMA MEMBRANE AND SERUM PROTEINS

A thesis submitted to the University of Surrey
for the degree of Master of Philosophy

by

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TO MY FAMILY
SUMMARY

The serum of tumour-bearing rats has been tested to determine if there is any protein shedding from the plasma membrane of the tumour cells. Both immunological and enzymological approaches have been employed. Methods for isolation of plasma membrane fragments for two different hepatomas have been developed. Plasma membrane fragments from the less rapidly growing (WDA) hepatoma are recovered in two distinct size classes which, it is suggested, derive from two distinct areas of the plasma membrane which have been distinguished by electron microscopy. Plasma membranes from the more rapidly growing (UA) hepatoma appeared much more uniform both in morphological appearance and in cell fractionation experiments. An antiserum has been prepared against purified plasma membrane fragments from the 'UA' hepatoma. The reaction of this antiserum has been examined by crossed immunoelectrophoresis and the various proteins characterised by comparison with the reaction of the sera with anti-(rat serum) and antiserum prepared against the serum of tumour-bearing rats and normal liver plasma membrane. The results did not show extensive release of material from the tumour plasma membrane into the serum, although evidence was obtained for a complex containing material deriving from tumour plasma membrane together with host serum proteins.

Enzymological methods have been used to study the 5'-nucleotidase and alkaline phosphatase enzymes in plasma membrane fragments and in serum. Evidence was obtained that the apparent 5'-nucleotidase activity of normal rat serum is largely due to a non-specific phosphatase. This was also suggested by gel filtration of serum on Sephadex G-200.
Gel filtration of the serum of tumour-bearing rats did, however, give evidence for the presence of a very high molecular weight complex which contains 5'-nucleotidase but with little if any alkaline phosphatase activity. This would be consistent with the complex deriving from the surface of tumour cells.
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<td>AFP</td>
<td>alpha fetoprotein</td>
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<tr>
<td>AMPase</td>
<td>5'-nucleotidase</td>
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<tr>
<td>Alk.PNPPase</td>
<td>alkaline phosphatase assayed with p-nitrophenyl-phosphate as substrate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>G.6.Pase</td>
<td>glucose 6-phosphatase</td>
</tr>
<tr>
<td>INT</td>
<td>2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltertrazolium chloride</td>
</tr>
<tr>
<td>PBS</td>
<td>physiological phosphate buffered saline</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
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<tr>
<td>Revs/min</td>
<td>revolutions per minute</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloracetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl) amino methane</td>
</tr>
<tr>
<td>TSTA</td>
<td>tumour specific transplantation antigen</td>
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CHAPTER ONE

INTRODUCTION
INTRODUCTION

The plasma membrane, which forms the surface of the animal cell, plays an important function in interactions between cells, in the response of the cells to the external environment and in the recognition of neighbouring cells. The immunological system of animals has the ability to reject any 'foreign' material to which it may have been exposed. It has been suspected that animals are capable of recognising at least some tumours as 'foreign' and should therefore be capable of rejecting them. However, many tumours grow and spread in the body. This is partly because the tumour-specific antigens are not strongly antigenic and the degree of immunity developed is insufficient to cause the rejection of a rapidly growing tumour, but the lack of rejection could also be explained by some material in the host organism which interacts with its defence mechanism. One suggestion is that so-called blocking antigens bind to circulating antibodies and so prevent them from reaching the site of the tumour. The interaction between tumour-specific antigens and antibodies will occur on the outer surface of the plasma membrane.

The discussion in the preceding paragraph shows clearly that it is important for cancer biochemists to understand the structure and function of the plasma membrane. Moreover, as the majority of the proteins present on the outer surface of the plasma membrane are glycoproteins, they contain carbohydrate attached by covalent linkages to the polypeptide chains. I shall discuss briefly the biosynthesis of glycoproteins and how these are transferred to the outer surface of the plasma membrane, before discussing the changes in the cell surface between cells in normal and neoplastic tissues.
The plasma membrane forms the barrier between the interior of the cell and the environment, and is the site at which external regulators such as hormones act on the cell. On examination under the electron microscope, the plasma membrane appears to be a three-layered structure with a total thickness of 75-100 Å, which is referred to as a 'unit membrane' (Novikoff, 1976). The structure and function of the plasma membrane has been recently reviewed by De Pierre and Karnovsky (1973), Harrison and Lunt (1975) and Finean et al. (1978). The fundamental function of the plasma membrane is that of protection. Thus the cell can maintain a constant internal environment, irrespective of changes that may occur outside. The plasma membrane has associated with it a range of 'transfer systems', which enable molecules to pass through the membrane in a specific manner. So the cell membrane, while protecting the cell from a variable external environment, allows selective communication with the exterior. Arrangements must be made for the controlled passage of nutrients into the cell, and the removal of waste products from it. 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. Finally, the plasma membrane may be involved in such sophisticated processes as immunological defence and information storage.

The plasma membrane contains lipids, proteins and carbohydrates. The carbohydrate is covalently bonded either to lipid or to protein, and the carbohydrate-containing molecules will be considered as lipids or proteins as appropriate.
hydrophilic head group within the molecule. Most of the polar lipids of cells are, in fact, localised in the membranes. Membrane lipids are organised as a bilayer. Membrane proteins show a complex distribution, some being attached to one or the other surface of a membrane and others either partially penetrating or passing right through a lipid bilayer. There are two classes of polar lipids, phospholipids and glycolipids. The lipids are arranged so that the long aliphatic chains are located within the interior of the membrane, and all the hydrophilic head groups face the aqueous environment.

Membrane proteins have been classified into two categories based on their ease of dissociation from the membrane, namely extrinsic proteins (also referred to as peripheral or membrane-associated proteins) and intrinsic (or integral) proteins. Currently accepted models of membrane structure distinguish between the locations of extrinsic and intrinsic membrane proteins. Both are believed to be globular proteins which are positioned differently relative to the lipid bilayer. Intrinsic proteins penetrate, and occasionally cross, the hydrophobic interior of the membrane, whereas extrinsic proteins are believed to be associated with the polar head groups of the lipid bilayer.

1-2 BIOSYNTHESIS AND SECRETION OF CELL COAT GLYCOPROTEIN

At the present time, there is great interest in the biosynthesis and function of membrane glycoproteins, particularly those associated with the cell surface, because evidence is accumulating that these compounds may be involved in cell-cell interactions. There are many reviews dealing with the biosynthesis of glycoprotein (e.g. Gottschalk, 1966; Nigam and Cantero, 1973; Lennarz, 1975). Glycoproteins are defined as protein-carbohydrate complexes in which oligo- or polysaccharides are joined by covalent linkage to specific amino acids of
proteins. The carbohydrate portion contains acidic or basic sugars (glucosamine, galactosamine, or sialic acid), and neutral sugars (galactose, mannose, or fructose).

The polypeptide components of glycoproteins are synthesised on ribosomes, bound to 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 Golgi apparatus plays an important role in the secretion of proteins and polysaccharides. The completed glycoprotein is then rapidly transferred to the outer surface of the plasma membrane to be added to the 'cell coat' (Bennett and Leblond, 1970).

Both N-acetylglucosamine and mannose are added to the polypeptide chain in the rough endoplasmic reticulum. It appears that mannose incorporation occurs immediately after the nascent protein is released from the polysomes, and that the released glycoprotein remains attached to the membrane of the endoplasmic reticulum (Redman and Cherian, 1972). When the newly formed glycoproteins pass to the smooth endoplasmic reticulum and the Golgi apparatus, other moieties of N-acetylglucosamine and galactose are incorporated into the glycoprotein side chains. At this point, the glycoprotein leaves the membrane and is found, together with albumin, within
the cisternae of the smooth microsomes (Redman and Cherian, 1972). A more detailed account of the biochemistry of glycoproteins can be found in "Glycoproteins" by Gottschalk (1972), as well as the review by Hughes (1975).

I-3 ALTERATIONS OF THE PLASMA MEMBRANE IN NEOPLASIA

Malignant tumours are characterised by abnormalities in the regulation of their growth and by their invasiveness. Therefore alterations in the cell surface membrane could be a crucial event in the transformation of normal cells into tumours. Hence, there have been, for many years, extensive studies on the surface of normal and tumour cells (e.g. Coman, 1960; Abercrombie and Ambrose, 1962; Emmelot and Benedetti, 1967; Wallach, 1969; Hakamori, 1973; Baldwin, 1976; Weinstein et al., 1976). A number of differences have been noted, and may be classified as follows:

(a) Alterations in cellular contact.
(b) Alterations in surface charge.
(c) Alterations in permeability.
(d) Alterations in the immunological properties of the cell surface.

(a) Alterations in cellular contact

(i) The alterations in intercellular adhesion

There is evidence that, in many tumours, the adhesion of neoplastic cells to each other and to normal cells is defective. Coman (1944) interpreted this evidence in terms of the decreased
'mutual adhesiveness' of the neoplastic cells, and correlated it with the impairment of the capacity of the cancer cell to bind calcium (Coman, 1953). He concluded (Coman, 1960) that, during the transformation of a normal to a neoplastic cell, an alteration of the external membrane takes place. Wallach (1969) also discusses changes in cellular adhesiveness during neoplastic conversion and quotes evidence showing that both increased and decreased mutual adhesiveness can occur. Intercellular adhesion in normal tissues as well as tumours is clearly a multifactorial process (Curtis, 1973), but there are nice reviews which deal with the topic (e.g. Curtis, 1973; Weinstein et al., 1976).

(ii) Contact inhibition of movement

This well known effect was extensively described by Abercrombie and Heaysman (1953) and Abercrombie and Ambrose (1958). They observed that cell locomotion appears to be inhibited when cells collide with their neighbours. Stoker (1967) has concluded that contact inhibition is commonly abnormal in tumour cells and discussed the fact that the defect appears very early in the neoplastic conversion of various cells by oncogenic viruses. However, some tumour cells, which are not inhibited by association with like cells, are inhibited by contact with normal cells or different tumour cells (Stoker, 1967). He explains these diverse observations by proposing that contact inhibition requires both a transmitter and a receptor and that the tumour cell can receive a specific message leading to contact inhibition but that it cannot send this message to a cell of its own kind.

Contact inhibition of movement has been given a simple definition
by Abercrombie (1970) as the stopping of the continued locomotion of a cell in the direction which has produced a collision with another cell. Weinstein et al. (1976) mention that many strains of malignant cells in culture are relatively insensitive to contact inhibition. However, this characteristic cannot be considered an invariant property of all malignant cells. More details and some theories attempting to identify a simple mechanism of contact inhibition of movement can be found in the review of Weinstein et al. (1976).

(iii) Electrical coupling and molecular transfer

It has been well established that electrical connections can occur between individual cells of various tissues (Loewenstein et al., 1965), including hepatocytes of normal or regenerating liver (Penn, 1966; Loewenstein and Penn, 1967). However, Loewenstein and his colleagues provided the first evidence of a defect in low-resistance coupling in solid tumours. They made electrical measurements on carcinomas in the liver (Loewenstein and Kanno, 1967), thyroid (Jamakosmanovic and Loewenstein, 1968), and stomach (Kanno and Matius, 1968), and found an absence of electrical coupling. Loewenstein proposed as the basis of these observations and theoretical considerations that a genetically determined interruption of junctional communication may be one of many causes of cancerous growth. Later, Sheridan (1970) succeeded in demonstrating electrical coupling between some tumour cells. This was confirmed by McNutt et al. (1971).

Communication between adjacent cells apparently acts as an important mechanism of metabolic control. Stoker (1967) showed that the defective nucleic acid metabolism of a mutant of polyoma
transformed cells can be corrected by cell contact with normal (unaffected) cells. This suggests that some undefined substance from the unaffected cell is passed to the mutant during cell contact.

(b) Alterations in surface charge

The interaction between individual cells has long been considered to depend on the charge density on cell surfaces (Wallach, 1969). Therefore many investigators have attempted to explain the altered contact behaviour of neoplastic cells by abnormalities of cell surface charge. However, as pointed out by Wallach (1969), no studies have been made correlating charge density, adhesiveness and contact inhibition. A number of workers (e.g. Forester et al., 1964) have suggested that the increased net surface charge of many neoplastic cells is due to an increased content of sialic acid (N-acetylneuraminic acid) in the plasma membrane. Mayhew (1966) has presented evidence that cell-surface sialic acid is synthesised during the late G₂ and mitotic phases of the cell cycle. Rosenberg and Einstein (1972) have confirmed this observation and suggested that an alternative interpretation is that the natural rate of destruction of surface sialic acid is decreased.

(c) Alterations in permeability

Alterations in the permeability of the plasma membrane of animal cells may play an important role in the regulation of cell growth and multiplication (Pardee, 1964; Sanford, 1967; Foster and Pardee, 1969; Weinstein et al., 19/6). There is evidence that the plasma membrane of tumour cells has abnormal permeability to certain intracellular enzymes, especially acid hydrolases (i.e. lysosomal) (see review by
Poole, 1973). It has been suggested that this phenomenon is closely related to the ability of many tumours to metastasise and to invade and destroy normal tissues (e.g. Carr, 1963; Weiss and Holyoke, 1969; Poole, 1973). The presence in the extracellular environment of tumours, of many normally intracellular enzymes, could be attributed to moribund or damaged tumour cells. However, in vitro studies on a variety of malignant cells have shown that viable cells may release large amounts of enzyme, apparently by active secretion in some cases (Poole, 1973). The mechanisms for this release are not yet known.

(d) Alterations in the immunological properties of the cell surface

The abilities to invade and metastasise constitute the lethal properties of malignant neoplasia and are the two clinical features that distinguish malignant neoplasia (cancer) from neoplasia in general. A non-invasive, non-metastasising neoplasm, even if rapidly growing, is clinically benign, not malignant. For neoplastic cells to form an invasive, metastatic tumour, they may need to overcome the disadvantage of their unique antigenicity, that is, they must avoid or escape from the host immune defences (Wallach, 1975).

Immunological changes of the plasma membrane associated with neoplasia fall into three categories as follows:

(i) Tumour specific antigens
(ii) Embryonic antigens
(iii) Antigen deletion
(i) Tumour specific antigens

Since the observation by Foley (1953) that tumours possess antigens which can elicit an immune response from the host, there have been extensive studies on the nature of these 'tumour-rejection' antigens (Wallach, 1969; Baldwin, 1973). The tumour-specific antigens detected in many carcinogen-induced tumours have been identified by their capacity to elicit immune reactions either in the original host against primary growths or in syngeneic hosts against transplanted tumour cells (Baldwin, 1973).

Tumour-associated rejection antigens are almost certainly located within the cell surface membrane. Perlmann and Holm (1969) have mentioned that it is difficult to conceive how intracellular antigens could operate to produce rejection reactions, because the tumour cell membrane is largely impermeable to antibody. The importance of cell membrane-associated antigens has been more directly demonstrated by in vitro studies of the cytotoxicity of immune serum or sensitised lymphocytes for culture tumour cells (Baldwin, 1973), although the mode of action of sensitised lymphocytes is still not yet clearly defined. The general opinion is that cell-mediated immunity is of primary importance in the early response of the body to a tumour.

(ii) Embryonic antigens

A further group of antigens found in tumours but not in normal adult cells is due to genes normally expressed during embryonic differentiation but repressed in adult life (time-displaced-antigens). Baldwin (1973) has suggested that embryonic antigens found in tumours may result from gene depression, which may be either directly or
indirectly attributed to the action of the chemical carcinogen. These antigens are detectable by immune diffusion methods in soluble fractions from hepatoma homogenates and also are present in the serum of tumour-bearing hosts, so that they may be viewed as secretory products of the tumour cell (see Baldwin, 1973). He has argued that the embryonic antigens in rat hepatomas and sarcomas are present in the cytosol, and are not found on the plasma membrane to any marked extent. These findings distinguish the embryonic antigens from the tumour-specific antigens which are known not to be present in intracellular fractions from hepatomas and sarcomas (Baldwin, 1973).

(iii) Antigen deletions

The binding of many chemical carcinogens to DNA and RNA has been observed (Baldwin, 1973). This may result in mutation leading to the production of novel proteins or to the induction or repression of 'normal' genes. The production of new proteins and the induction of embryonic genes has been discussed above, but neoplastic transformation may also be accompanied by the loss of organ-specific antigens. While many of these deleted antigens are intracellular, rather than plasma membrane components, there is evidence that some plasma membrane antigens are lost during carcinogenesis. Baldwin (1973) has suggested that this loss of normal plasma membrane antigens may be causally related to the appearance of tumour-specific antigens, but it has not been established that these 'deleted antigens' are actually lost from the plasma membrane. It is possible that the antigens may simply be located at some site in the plasma membrane of tumour cells that is inaccessible to antibodies.
There are two different types of immunological response which occur when 'foreign' antigens enter the body.

(i) Free antibody is synthesised and released into the blood and other body fluids. This type of immunological response is called a 'humoral response'.

(ii) The second type is 'cell-mediated immunity' by production of sensitised (T) lymphocytes which have antibody-like molecules on their surface (cell-bound antibody) (see Roitt, 1977).

If (T) lymphocytes recognize tumour antigens as foreign, they may develop into killer cells and/or they may stimulate other cells to become killer cells, both specifically, e.g. by producing antibodies that can 'arm' bone marrow-derived (B) lymphocytes and macrophages, and nonspecifically by releasing various substances that can activate other cells as well as help to destroy the tumour by a cytotoxic effect (Hellström and Hellström, 1974).

Immunological recognition of malignant cells is clearly important in the tumour-host relation. There is clear evidence that many tumours are recognised as 'foreign' and the neoantigens involved have been characterised in a number of animal tumours (Baldwin, 1973; Lamon, 1974; Baldwin and Price, 1975). However, it is clear that tumour-associated antigens expressed at the cell surface do not necessarily function as effective rejection antigens, even though they elicit specific immune responses in the tumour-bearing host. For example, Baldwin and Robins (1976) have pointed out that in
chemically induced tumours, the tumour rejection antigens are often characteristic components of individual tumours so that tumour immunity is effective only against cells of the immunising tumour.

Several explanations can be proposed to account for the failure of tumour-associated cell-surface antigens to mediate tumour rejection responses including:

(i) immunodeficiency of the host;
(ii) sneaking through;
(iii) alteration in neoantigen expression and/or function;
(iv) abrogation of tumour immunity by host factors.

The concept that tumour-associated antigens may not be recognized by the host has proved generally untenable, in view of the many studies demonstrating cellular and humoral immune responses in tumour-bearing individuals (Hellström and Hellström, 1974). The 'sneaking-through' hypothesis implies that a nascent tumour may not provide sufficient stimulus to induce an effective immune response, so that, when host defences are eventually mobilised, the tumour is already of a size that is outside immunological control (Baldwin and Robins, 1976).

Tumour cells undoubtedly show considerable variation in the degree of neoantigen expression, so that whilst they may provoke an effective immunological response, subpopulations of tumour cells may not be susceptible to immune rejection reactions. Baldwin and Robins (1976) have explained that this may result from quantitative differences between subpopulations of tumour cells in neoantigen expression. Alternatively, the defect may be functional, in that,
whilst neoantigens continue to be expressed at the tumour cell surface, they are inadequate receptors for rejection reactions mediated by sensitised lymphoid cells or antibody. Baldwin and Robins (1976) have shown that one factor important in this respect is the degree of integration of tumour antigens in the cell-surface membrane. Baldwin et al. (1974) have already indicated that embryonic antigens associated with carcinogen-induced and spontaneous rat tumours do not function effectively, if at all, in tumour rejection responses; these antigens are poorly integrated components of the cell surface, which are readily released on cell rupture. The final hypothesis put forward by Baldwin and Robins (1976) proposes that failure of immune responses in the tumour-bearing host to control tumour growth results from the intervention of host factors that diminish the effectiveness of cell-mediated immunity. These factors may operate independently or in concert to abrogate immunological control of an early developing tumour.

**Humoral factors modifying cell-mediated immunity in the tumour-bearing host**

(a) **Blocking reactions at the tumour cell surface**

Interference with cell-mediated immunity in the tumour bearing host may, in certain circumstances, be produced by serum factors interacting with and masking neoantigens on tumour cells, so preventing their recognition by sensitised lymphoid cells; this has been defined operationally as 'blocking' (Baldwin and Robins, 1976).
(b) **Inhibition of lymphoid cell reactivity**

An alternative pathway by which serum factors in the tumour-bearing host may abrogate cell-mediated immune rejection reactions involves specific desensitisation (inhibition) of cytotoxic lymphoid cells through interaction with tumour antigen either in the free form or as immune complexes.

Circulating tumour antigen and/or immune complexes have been implicated in the inhibitory type of response which leads to a specific reduction in the reactivity of sensitised lymphocytes, since serum activity rapidly decreased in animals rendered tumour-free (Robins and Baldwin, 1974; Shellam and Knight, 1974). Robins and Baldwin (1974) have provided further support for the concept that tumour antigen-containing factors in tumour-bearer serum inhibit tumour-immune lymphocyte cytotoxicity by studies on the neutralisation of this activity by tumour-specific antibodies. Antibody neutralisation of lymphoid cell inhibitory factors in tumour-bearer serum leads to the conclusion that circulating tumour-specific antigen and/or immune complexes with available antigen receptors are involved in these interactions.

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**I-5 GENERAL METHODS FOR THE ISOLATION OF PLASMA MEMBRANE FRAGMENTS FROM NORMAL AND NEOPLASTIC TISSUES**

The isolation of plasma membrane fractions from either normal or neoplastic tissues has been the subject of several extensive reviews (Steek and Wallach, 1970; Eylar and Hagopien, 1971; Warren and Glick, 1971; Hinton, 1972; De Pierre and Karnovsky, 1973). In any cell
fractionation technique, the aim is to separate a particular cell component intact and undamaged. In the case of 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 by intracellular membranes. Isolation of plasma membrane may conveniently be discussed under five headings:

- the nature of the tissue to be used,
- the selection of markers,
- disruption of the tissue,
- fractionation,
- analysis

(De Pierre and Karnovsky, 1973). In the case of solid tissues such as liver, the cells are bound to each other and form junctional complexes (tight junctions - zonula occludens, intermediate 'gap' junctions - zonula adherens, desmosome junctions - macula adherens). These appear to remain intact during homogenisation of the liver, and as a result the plasma membrane from the zones containing the complexes is released as large sheets, deriving from several adjacent cells (Hinton et al., 1970), which sediment into the crude nuclear fraction. Plasma membrane fragments are also recovered in the microsomal fraction as vesicles (El-Aaser and Reid, 1969; Wisher and Evans, 1975). These possibly derive from regions of the plasma membrane other than the junctional complexes.

(a) Separation of large sheets of plasma membrane

Most procedures for the isolation of plasma membrane from solid tissues aim at separating the large sheets of membrane, which sediment
in crude nuclear fraction. All the methods for preparing large sheets of plasma membrane fragments from liver are based on the method of Neville (1960), which was modified by Emmelot and his colleagues (Emmelot et al., 1964). This method consists of gentle homogenisation in hypotonic NaHCO₃ (1 mM) and preparation of a low speed pellet which is then separated and carefully washed to remove mitochondria and other organelles. Strongly hypotonic solutions are required in order to lyse the erythrocytes, which otherwise aggregate with the membrane (Hinton et al., 1970); the use of such solutions prohibits the isolation of other organelles in an intact state. A better method for the removal of erythrocytes than homogenisation in hypotonic media is perfusion of the tissue in situ (Coleman et al., 1967; Hinton et al., 1970). One can then homogenise in isotonic solutions.

In most tumours perfusion is impossible because of the lack of a main blood vessel to the tumour. Therefore, Emmelot and his colleagues (Emmelot and Bendetti, 1967; Emmelot and Bos, 1969) had recourse to very hypotonic solutions for the isolation of plasma membrane from hepatomas. However, there are two further problems with hepatoma: the first is that the nuclei of many tumour cells are extremely fragile, and nucleoprotein gels may prevent separation of cell components from homogenates prepared in hypotonic solutions (Davydova, 1968; Prospero, 1975). The second problem is that the weak intercellular bonds in many tumours result in the tissue initially separating into single cells on homogenisation and yielding small sheets and vesicles. However, Prospero and Hinton (1973) showed that the use of zonal rotors allowed the preparation of highly purified plasma membrane from a slowly growing transplanted hepatoma and this method has been further developed in this study.
(b) Isolation of plasma membrane vesicles from the microsomal fraction

Plasma membrane vesicles are very similar in size and density to vesicles deriving from other organelles. Because of the difficulty of separating such vesiculated plasma membrane directly from microsomal fractions, methods have been developed which depend on selectively modifying the density of some of the vesicles. Kamat and Wallach (1965) were the first to attempt the separation of the microsomal plasma membrane from the bulk of the microsomes (e.g. the endoplasmic reticulum fragments), with Ehrlich ascites carcinoma cells as the test material. They subfractionated microsomes by isopycnic centrifugation on Ficoll gradients and found the plasma membrane at a median density of 1.07. Flotation in sucrose gradients has been used to emphasise the difference in the density of hepatic plasma membrane vesicles from those deriving from other organelles (Touster et al., 1970; Hinton et al., 1971; Norris, 1973) with varying success. Touster et al. (1970) used a discontinuous sucrose density gradient buffered with 5 mM tris-HCl, pH 8.0, for preparation of microsomal plasma membrane. They recovered the plasma membrane as a turbid white band of material of density 1.15 with relative specific activities of 5'-nucleotidase and phosphodiesterase about twenty times that of glucose-6-phosphatase.

In general, most workers have preferred to isolate plasma membrane fragments in the form of sheets rather than as vesicles. Prospero (1975) has suggested that is not only because of the difficulty of suitably subfractionating microsomal fractions, but probably also due to the reliance on morphological as distinct from biochemical identification shown by many investigators. The results of those
investigators who have studied the distribution of the plasma membrane marker enzymes in the microsomal fraction will be discussed further, together with my own results (Chapter IV-1).

I-6 BIOCHEMICAL MARKERS FOR HEPATOMA ORGANELLES

Obviously, when one wishes to isolate one component from a mixture, it is necessary to have assays both for that component and for other components which may contaminate the preparation. In this study, biochemical criteria, i.e. the assay of marker enzymes, have been used to identify the various subcellular organelles. The use of marker enzymes in tissue fractionation studies has been the subject of several recent reviews (e.g. Reid, 1971), while other authors (e.g. Steck and Wallach, 1970; Solyom and Trams, 1972; De Pierre and Karnovsky, 1973) have discussed the particular use of marker enzymes in the isolation of plasma membrane.

As pointed out by Reid (1967, 1971) the marker enzymes which are used in the case of normal liver do not necessarily hold true for other tissues, e.g. in muscles glucose-6-phosphatase is not available as a marker for endoplasmic reticulum as it is in liver. Similarly the ATPase of the plasma membrane is distinct from the ATPase of muscle sarcoplasmic reticulum and mitochondria. In general, enzymes reflect the function of the tissue. In particular, Prospero (1975) found that markers established for normal liver are not necessarily valid for hepatomas under standard assay condition.

The enzymes considered in this study as markers for specific organelles were:
(i) 5'-nucleotidase for plasma membrane fragments.
(ii) Glucose-6-phosphatase for endoplasmic reticulum.
(iii) Acid phosphatase for lysosomes.
(iv) Succinate dehydrogenase for mitochondria.

(i) 5'-nucleotidase

The location of this enzyme in tumour was examined by a cytochemical technique (Fitzsimmons, 1969). An activity was found in the plasma membrane, but not in other organelles. He observed that in the transplanted hepatoma, the cellular localisation of 5'-nucleotidase is similar to that in normal liver (El-Aaser, 1965). The distribution of 5'-nucleotidase among hepatoma cell components was used by Emmelot and Bos (1969) and Prospero (1975) as a marker for the plasma membrane fragments. Consequently, 5'-nucleotidase was used in this work as a marker for the hepatoma plasma membrane fragments.

(ii) Glucose-6-phosphatase

It has long been known that, after fractionation, this enzyme is recovered in the microsomal fraction (e.g. de Duve et al., 1955). More recently its location in endoplasmic reticulum membranes has been shown cytochemically (see Reid, 1967). In the case of hepatoma, Fitzsimons (1969) studied the distribution of glucose-6-phosphatase activity using cytochemical techniques. He observed that a positive reaction is given only by endoplasmic reticulum and nuclear membrane. Prospero (1975) investigated the distribution of glucose-6-phosphatase activity among subfractions separated from hepatoma mitochondrial and lysosomal fractions using an HS zonal rotor. He observed that 10 mM Na(+) tartrate reduced the activity in the homogenate by 50-60%.
Na(+) tartrate does not inhibit liver glucose-6-phosphatase but does inhibit lysosomal acid phosphatase. Hence Prospero (1975) concluded that there was, in hepatoma homogenates, significant breakdown of glucose-6-phosphate by acid phosphatase. So, in this study glucose-6-phosphate assayed in the presence of 10 mM Na(+) tartrate to inhibit acid phosphatase, was used as a marker for the endoplasmic reticulum membranes.

(iii) Acid phosphatase

The distribution of acid phosphatase (using β-glycerophosphate as the substrate) is considered a reliable indicator of the distribution of lysosomes (De Pierre and Karnovsky, 1973). Burge (1973) found that the distribution of acid phosphatase in the hepatoma lysosomal fraction parallels that of the other acid hydrolases, and concluded that acid phosphatase is a suitable marker for lysosomes. Therefore, this enzyme was used during the course of my study to determine lysosomal contamination of the plasma membrane preparations.

(iv) Succinate dehydrogenase

This enzyme is the most common mitochondrial marker. de Duve (1967) has mentioned that the use of succinate dehydrogenase as a marker for mitochondria is extremely well established. A dye (INT) is a very convenient acceptor in the assay of this enzyme (Pennington, 1961). In this study succinate dehydrogenase was used as a marker for mitochondria.
THE AIMS OF THIS STUDY

The plasma membrane (animal cell surface) plays an important function in interactions between cells, in the response of cells to the external environment and in the recognition of neighbouring cells. The immunological system of animals has the ability to reject any strange body to which it may have been exposed. However, growing tumours are not rejected although, at least in experimental animals, some tumours are recognised as 'foreign'. An important factor for the growth and spread of tumours in the body is that the tumour-specific antigens are often not strongly antigenic and the degree of immunity developed is insufficient to cause the rejection of a rapidly growing tumour. A further explanation is that some material in the host organism interacts with its defence mechanisms. One suggestion is that so-called blocking antigens bind to circulating antibodies or lymphocytes and so prevent them from reaching the site of the tumour. The interaction between tumour-specific antigens and antibodies will occur on the outer surface of the plasma membrane, so that one would expect 'blocking factors' to derive from the tumour plasma membrane.

The main objective of this study is to determine whether or not the plasma membrane is releasing these materials into the blood. I will describe experiments to show whether a method for isolating purified plasma membrane sheets from slow growing hepatoma, developed some years ago in this laboratory, is still applicable following progression of the tumour, and whether it can be used with another, more rapidly growing hepatoma. I will also describe the development of a method for isolating plasma membrane vesicles from the microsomal fraction of both tumours and the preparation of an anti-(plasma
membrane) antiserum using the purified plasma membrane separated from
the hepatoma. Immunological techniques are used to investigate the
antigens common to rat serum and hepatoma plasma and to study other
changes in serum proteins of tumour-bearing rats. Enzymological
methods are used to study the enzyme 5'-nucleotidase which is a marker
for plasma membrane fragments and is also present in serum.
Ouchterlony double diffusion techniques are used to find out if there
is any relation between the 5'-nucleotidase in hepatoma plasma membrane
and the plasma membrane of normal rat liver. I have also examined
the serum of tumour-bearing rats to determine whether there is any
evidence for release of enzymatically active material from the tumour
surface. It is hoped that a combination of immunological and
biochemical methods will give a clearer picture of the extent to which
tumours shed surface components into the blood stream.
CHAPTER TWO

MATERIALS AND METHODS
II-1 MATERIALS

In general, all chemicals were analytical grade, obtained from BDH Chemicals Ltd., Koch-Light Ltd., Hopkins and Williams Ltd. or the Sigma Chemical Co. Ltd. Distilled water was used in all media, and all pH measurements were made at the temperature of use for each solution, e.g. for enzyme assay buffers at 37°C.

Tris buffer [tris-(hydroxymethyl)-aminomethane], activated charcoal and bovine serum albumin were obtained from Sigma Chemicals Co. Ltd., AMP (adenosine-5'-monophosphoric acid(disodium salt)), G.6.P (glucose-6-phosphoric acid(disodium salt)) from BDH, INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride] and DMG β,β-dimethyl glutaric acid were brought from Koch-Light Ltd. Sodium β-glycerophosphate and bis(p-nitrophenyl)-phosphate(disodium salt) were obtained from BDH Chemicals Ltd. Mineral water sucrose was bought from Tate and Lyle Ltd. Sepharose-4B was obtained from Pharmacia Ltd. (UK). Anti-rat serum was obtained from Mercia Diagnostics Ltd (High Wycombe, Hertfordshire). All centrifugation was carried out at 4°C in MSE centrifuges.

II-2 ANIMALS AND TUMOURS

The animals used were hooded rats of the University of Surrey strain. The hepatomas were transplants from primaries induced, by Drs. E. Reid and B.M. Mullock, by feeding L-ethionine to male rats (Reid, 1970). Transplantation was by the subcutaneous route. Two distinct hepatomas were studied; the faster growing (UA) tumour has a generation time of 2-3 weeks and the slow growing (WDA) tumour a generation time of about 6 weeks.
All assay results were corrected for sucrose interference as described by Hinton and Dobrota (1969) and Hartman et al. (1974). In general, results were processed using a computer program developed by Hinton (unpublished method).

(a) Protein estimation

Protein was assayed by the Autoanalyser adaptation (Schuel and Schuel, 1968) of the method of Lowry et al. (1951), as modified by Hinton and Norris (1972). The "high sensitivity" manifold was used for measurements of the protein content of all fractions, with bovine serum albumin (BSA) standards ranging from 0.01 - 1 mg/ml.

(b) Enzymes releasing inorganic phosphate

The following enzymes were assayed by measuring the amount of phosphate released during the assay:

- 5'-nucleotidase
- Glucose-6-phosphatase
- Acid β-glycerophosphatase
- Alkaline β-glycerophosphatase
- Adenosine triphosphatase

In general, the assays for phosphatases were carried out as described by Prospero et al. (1973). The basic assay system was the same for all these enzymes. An aliquot of 0.5 ml of tissue was mixed with 0.4 ml buffer containing any necessary activators. The reaction was then started by the addition of 0.1 ml of substrate, followed by mixing on a Whirlimixer, and incubated at 37°C for a suitable period of time.
Due to the presence of some endogenous phosphate, substrate and tissue blanks were always used. Tissue blanks were performed, for each fraction, by adding 0.1 ml distilled water instead of the substrate. Substrate blanks were done, usually in duplicate, using 0.5 ml distilled water in place of the tissue sample. Assays on reference samples (homogenate, nuclear fraction, floated material from the whole homogenate and microsomal fraction) were normally done in duplicate.

The reaction was stopped by adding 1.5 ml of 6% w/v TCA and after a period at 4°C 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). The standards used were sodium dihydrogen orthophosphate solutions (0.1 - 1 μ moles PO₄/μl), and treated as the samples, e.g. 1.5 ml trichloroacetic acid was added per 1 ml of the standard.

The validity of the assays for the following enzymes: alk. PNPPase, alk. β-Gly.Pase and AMPase has been checked for serum samples. The linearity of each assay with incubation time was examined to determine the maximum time suitable for the assay (Figure II-1).

Activity of alk. PNPPase was also checked with different substrate concentrations, to establish the optimum substrate concentration which should be used (Figure II-2).

It was found that the assay, as used previously for tissue fractions, was valid for use with serum samples.

(c) Enzymes releasing p-nitrophenol

Enzymes releasing p-nitrophenol, namely alkaline phosphatase and alkaline phosphodiesterase, were assayed by the automated method of Hinton
FIG. II-1  Linearity of the assay of
(a) 5'-nucleotidase
(b) alkaline β-glycerophosphatase
(c) alkaline p-nitrophenylphosphatase
with incubation time
FIG. II-2 Activity of alkaline $\rho$-nitrophenylphosphatase in the serum of a normal rat as a function of substrate concentration.
and Norris (1972). Substrate concentrations were 0.01 M \( p \)-nitrophenyl phosphate for phosphatase, and 0.01 M bis(\( p \)-nitrophenyl)-phosphate for phosphodiesterase.

(d) **Succinate dehydrogenase**

Succinate dehydrogenase was assayed by the method of Pennington (1961). To an aliquot of 0.5 ml of tissue was added 0.25 ml of a freshly-prepared 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 min, the reaction was stopped by 1.5 ml of 6\% trichloroacetic acid. The red formazan was extracted into 4 ml of ethyl acetate which was then separated from the aqueous phase by a short spin. The colour of the extracts was measured at 490 nm against an ethyl acetate blank. Tissue blanks were determined by substituting 0.3 M malonate, pH 7.4 for the succinate in the assay medium.

**II-4 TISSUE PREPARATION**

(a) **Rat liver**

After killing the animal by cervical dislocation, the liver was removed and immersed in ice-cold 0.25 M sucrose containing 5 mM tris-HCl buffer, pH 8. The tissue was weighed and minced with scissors. Homogenisation was by three strokes of a Potter-Elvehjem homogeniser with a pestle rotated at 900 rev/min. The diametric clearance between pestle and vessel was 0.33 mm. The homogenate was filtered through a coarse sieve to remove any connective tissue fragments.
(b) Tumours

The animals were killed by cervical dislocation and the tumours rapidly extracted, complete with their capsule, and chilled by immersion after removing the necrotic centre in ice-cold sucrose. After weighing, the entire tumours were minced with scissors and homogenised with a Potter-Elvehjem homogeniser. The homogenate was filtered as described above, small pieces of connective tissue from the capsule being retained on the filter. The exact weight of tissue was obtained by subtracting the weight of the retained material from the weight of the whole tumour.

II-5 ZONAL CENTRIFUGATION

(a) Preparation of density gradients

Sucrose gradients were used in all the experiments with zonal rotors described in this study. Sucrose solutions were prepared from a stock solution of 2M sucrose. Their concentrations were checked with an Abbé refractometer (Bellingham and Stanley Ltd., London) at 20°C. Possible impurities were removed from the stock solution by stirring with Norit 'A' activated charcoal (34 g/l) for about 30 min (Steele and Busch, 1967). The charcoal was removed by filtration through Whatman no.54 filter paper.

There were two types of gradient used:

(i) An "exponential" gradient, used in all experiments with the H-S zonal rotor.

(ii) A "linear" gradient, used with the B-XIV zonal rotor, which was generated with the "double pump" gradient maker described by Hinton and Dobrota (1969).
(i) Preparation of exponential gradients

The apparatus used for the preparation of this type of gradient is shown in Figure II-3a. Initially 150 ml of 0.35M sucrose (density 1.045 g/ml) was placed in the mixing vessel and put on a magnetic stirrer. The following sucrose solutions were added:

<table>
<thead>
<tr>
<th>Gradient H-S</th>
<th>Vol ml</th>
<th>Sucrose conc. M</th>
<th>Density g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>The mixing vessel initially contained</td>
<td>150</td>
<td>0.35</td>
<td>1.045</td>
</tr>
<tr>
<td>To this was added</td>
<td>300</td>
<td>0.95</td>
<td>1.124</td>
</tr>
<tr>
<td>The mixing volume was then reduced to (by decantation - with the pump switched off)</td>
<td>75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>To this was added</td>
<td>200</td>
<td>1.31</td>
<td>1.170</td>
</tr>
<tr>
<td>Cushion (by-passing mixing vessel) to fill rotor</td>
<td>2.00</td>
<td>1.249</td>
<td></td>
</tr>
</tbody>
</table>

(ii) Preparation of linear gradient

The gradient maker used for this preparation is shown in Figure II-3b. A double piston pump with adjustable flow rate on each channel was used to prepare the gradients. The mixing vessel, put on a magnetic stirrer, initially contained 375 ml of 0.6M sucrose (density 1.077 g/ml). Liquid was pumped out from the mixing vessel into the zonal rotor at 40 ml/min, while 2M sucrose was being added at 20 ml/min, until the whole solution from the mixing vessel was used. The density of the resulting gradient was found to range from 1.077 - 1.257 g/ml.

(b) Loading and displacement of the gradients

Linear or exponential gradients, prepared as described above, were loaded into the zonal rotor. Usually the refractive index of the gradient pumped into the rotor was recorded as a check on the shape of the gradient. With the HS rotor, after the exponential gradient had been loaded, the sample was injected carefully through the central line of the
FIG. II-3  (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
feed head. An overlay of 0.08M sucrose was loaded to the centre to displace the sample away from the core of the rotor; then the rotor was accelerated from the speed of loading (1,200 revs/min) to a high speed (9,000 revs/min). With the B-XIV rotor, on the other hand, the sample, which was made up in 2M sucrose, was injected to the edge of the rotor at the dense end of the linear gradient, and, to serve as a cushion, 2M sucrose was pumped immediately after the sample. The speed of the rotor during the loading of gradient and the sample was about 2,000 revs/min; when loading was completed, the lid of the centrifuge was closed and the rotor accelerated to 44,000 revs/min. After centrifugation for the required time, the rotor was decelerated to the loading speed and the contents unloaded by pumping in 2M sucrose to the edge of the rotor.

II-6 ELECTRON MICROSCOPY

Tissue was prepared for electron microscopy by a procedure essentially similar to that of El-Aaser (1965). After rapid removal of the tumour from the rats, samples were prepared by chopping the tumour into 1 mm cubes on a cooled glass plate. The tissue was first fixed in ice-cold 1% glutaraldehyde (Taab Laboratories Ltd.) dissolved in 0.25M sucrose buffered with 0.05M sodium cacodylate, pH 7.2, for about 2h. The blocks of tissue were washed with 0.094 cacodylate, pH 7.4, and then immersed in 2% osmic acid (Taab Laboratories Ltd.) for about 1h, and dehydrated (twice) by 10 min periods of exposure to 10%, 25%, 50%, 75%, 90% and 100% absolute ethyl alcohol. It was then immersed in propylene oxide for 10 min (twice) and in a mixture of propylene oxide + Epon 812 (Taab Laboratories Ltd.) for 30 min. Then the blocks were embedded in Epon and put in the oven for 48 h at 60°C to polymerise the sample. After that very thin sections were cut, counterstained with alkaline lead
citrate and uranyl acetate and examined using a JEM 100B electron microscope.

II-7 PREPARATION OF SEPHADEX GEL COLUMNS

Gels were allowed to swell in excess 0.9% sodium chloride. The initial swelling of the gel slurry was performed on a boiling water bath. This reduces the number of air bubbles trapped in the slurry as these may cause uneven flow rates in the packed columns.

Columns were packed in either vertical glass tubes with a sinter-glass disc fused across one end to support the gel or in the special Sephadex laboratory columns (Pharmacia, Sweden). The columns were packed as recommended by Pharmacia Ltd. The column was filled with the Sephadex slurry and gently stirred to remove any air bubbles. The outlet was opened and positioned to allow the solvent to flow out slowly while the remaining slurry was slowly added to the column. The addition of slurry was continued until the required bed height was obtained. The upper surface of the bed was protected by a circular piece of filter paper. A solvent reservoir was connected to the top of the column and the flow rate maintained at a rate of approximately 0.35 ml/min.

The void volume of the column was measured by noting the volume of effluent between loading Blue Dextran 2000 (Pharmacia) on the top of the column and its appearance at the end of the column. The sample solution was applied to the top of the column by Pasteur pipette, without disturbing the surface of the column. The density of the sample solution was increased by the addition of sucrose (about 30 mg/ml). Once the sample had loaded the column was filled and connected to an eluent reservoir fitted with a constant head device. Fractions were collected
by time, using a timer connected to a fraction collector (Central Ignition Co.).

Although the first experiments were carried out using the technique above, the majority of separations were performed by upward displacement using large column made by my colleague, Dr. B.M. Mullock. The columns were prepared as described by Fischer (1969). Columns used for upward displacement are more difficult to prepare as air bubbles must be rigorously excluded. Hence, it is usual to use each column repeatedly.

To prevent bacterial growth the columns are equilibrated with 0.002% hibitane between experiments. Prior to each of my experiments, I washed the column extensively with 0.9% NaCl to remove all traces of hibitane.

Total volume ($V_t$) of the gel was calculated from the dimensions of the column. The void volume ($V_v$) was determined as mentioned above. The elution volume ($V_e$) for a specific peak was measured for each sample. Hence the partition coefficient for a particular sample peak between the mobile liquid phase and the stationary gel phase may be determined from the formula:

$$K = \frac{V_e - V_v}{V_t - V_v}$$

### II-8 GEL ELECTROPHORESIS

For all immunoelectrophoresis techniques described in this study, 1% w/v agarose in 0.044M-barbitone buffer, pH 8.6, was used. The agarose plates were prepared by pouring 10 ml of molten agarose on to an $8 \times 8$ cm glass plate, giving 3 mm gel thickness. After cutting and clearing the wells, a drop of the antigen solution was loaded and electrophoresed at room temperature for about 1h at 10 V/cm.
After running, the agarose was cut 5 mm from the centre of the well. The strip containing the sample was retained whilst the rest was removed and discarded. For the second dimension, agarose containing antibodies was poured onto the remaining part of the plate and electrophoresed for about 18 h at 2 V/cm. After running, the non-precipitated proteins were removed by pressing the plates and washing out with 0.9% NaCl and with distilled water. The plates were then dried and stained for protein with Coomassie Brilliant Blue R-250 in 9:9:2 ethanol:water:glacial acetic acid (Axelsen et al., 1973) or with Sudan Black-B for lipid-containing lines (Clausen, 1969).

Polyacrylamide gel electrophoresis was performed in 8 cm x 5 mm glass tubes using a home-made electrophoresis apparatus. The stock solutions required were made as follows:

**Acrylamide:** 30 g acrylamide + 0.8 g bis-acrylamide dissolved and diluted to a final volume of 100 ml with distilled water. The solution was filtered if cloudy.

**Tris pH 8.9:** 36.3 g of tris base + 48 ml N\textsubscript{H}Cl dissolved and made up to 100 ml with distilled water. (As with any buffer recipe, the correctness of the pH was verified.

**Tris pH 6.7:** 5.98 g of tris base dissolved in distilled water and adjusted to pH 6.7 with HCl and, finally, made up to 100 ml with distilled water.

**10% SDS:** 10 g of sodium dodecyl sulphate made up to 100 ml with distilled water. SDS solutions precipitate in the cold, but redissolve immediately on warming.

**TEMED:** N,N,N',N'-tetramethylethylenediamine.

**Persulphate:** 10 g sodium persulphate per 100 ml.
Electrophoresis buffer (pH 8.9): 12 g tris base + 57.6 g glycine made up to 21 with distilled water. Add 10% SDS before use, 10 ml/l of buffer.

Membrane proteins were dissolved by heating for 3 min at 100°C in the following mixture:

1 ml tris pH 6.7
0.9 ml glycerol
0.01 ml mercaptoethanol
0.2 ml bromphenol blue (amount should be adequate to give a strong colour)
4.5 ml 10% SDS
Add water (2.4 ml) to make up to 9 ml.

Then the polacrylamide gels were prepared as follows:

5 ml acrylamide
1.88 buffer (tris-glycine) pH 8.9
0.15 ml 10% SDS
0.0075 ml TEMED
7.9 ml distilled water
0.075 ml freshly prepared ammonium persulphate

The tubes were filled with these solutions up to 7 cm height and carefully overlaid with distilled water. When these gels were set, the water was removed and spacer gels were loaded, prepared from the same solutions as follows:

0.5 ml acrylamide
0.625 ml tris buffer pH 6.7
0.05 ml 10% SDS
0.0025 ml TEMED
3.77 ml distilled water
0.05 ml ammonium persulphate
These were then overlaid with distilled water as before. After polymerisation (about 20 min), samples were layered on top of the polyacrylamide. Electrophoresis was done for about 3h at room temperature in tris glycine buffer pH 8.3 (see above for details) using 5 mA/tube. Then the gels were stained for protein with Coomassie Brilliant Blue-G overnight which was prepared as below; then destained with several changes of distilled water till a clear background was obtained (Blakesley and Boezi, 1977).

0.2% aqueous solution
Add equal volume of 2N H₂SO₄
Stand for at least 3h
Filter through Whatman No.1
Add 1/9 volume (accurate) of 10N KOH
Add 100% w/v TCA to give final concentration of 12%

II-9 RAISING OF ANTI-(SERUM OF TUMOUR BEARING RATS) AND ANTI-(HEPATOMA PLASMA MEMBRANE) ANTIBODIES

Two rabbits were used to raise antibodies against serum of tumour bearing rats and hepatoma plasma membrane fragments. The plasma membrane used for immunisation was washed out with 0.15M-NaCl. 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 week intervals with 1.5 ml of the appropriate suspension. The suspensions were made up of 0.5 ml of a 6 mg/ml solution of antigen protein in physiological phosphate-buffer saline (0.15M-NaCl/0.01M-phosphate, pH 7.5) (PBS) and 1.0 ml of Freund's complete adjuvant for the first injection. Incomplete adjuvant was used for the rest. Each rabbit received four injections.
After four weeks, another injection at the same protein concentration was given.

After 8 days, the rabbits were bled. The serum was separated 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 0.15M-NaCl to give a final volume of one-third that of the original serum. The solution was then dialysed against several changes of 0.15M-NaCl at 4°C for at least 24h. The diffusate was tested with barium chloride to ensure the complete removal of the sulphate.
The sera of normal and tumour-bearing rats were fractionated by three different techniques: gel filtration, immuno-electrophoresis and polyacrylamide gel gradient electrophoresis.

(a) Gel filtration technique

(i) By using Sephadex G-200

The column of Sephadex G-200, prepared as discussed in Section II-7, was used to separate serum proteins on the basis of their molecular size. Large molecules, which cannot penetrate the pores of the bed molecules, pass through the column directly, while smaller molecules are retarded. The grade of Sephadex (G-200) used in this study can be used to fractionate globular proteins with molecular weights ranging between 5000-500000.

The distributions of the protein and various enzyme activities after gel exclusion chromatography of serum from (UA) tumour-bearing animals are illustrated on Fig. III-1, while those for normal serum (an experiment carried out by my colleague, Dr B. M. Mullock) are shown on Fig. III-2.

In the case of normal serum, all enzymes seem to run as a single, rather broad, band. Examination of the distribution of 5'-nucleotidase after fractionating the serum of the (UA) tumour-bearing rats, shows a small peak in the early fractions, suggesting that a small quantity of this enzyme is associated with large molecules, which pass through in the void volume. The main peak of 5'-nucleotidase is found in fraction No. 26. The main peak of alkaline β-glycerophosphatase
FIG. III-1  Distribution of protein and various enzymes after fractionating the serum of a 'UA' tumour-bearing rat on a 2.2 x 31.5 cm column of Sephadex G-200. The column was eluted by upward displacement with 0.15 M NaCl and 3.5 ml fractions collected. The void volume of the column was 30 ml, corresponding to fraction No.'9'

(a) ——— protein

(b) □——□  alkaline β-glycerophosphatase

   ▼——▼  5'-nucleotidase

(c) ●——●  alkaline p-nitrophenylphosphatase

   △——△  alkaline phosphodiesterase
FIG. III-2  Distribution of protein and various enzymes after fractionating the serum of normal rats on Sephadex G-200, as described in the legend to Fig. III-1.

(a) __________ protein
    ●----●  5'-nucleotidase
    ▼——▼  alkaline β-glycerophosphatase

(b) ■——■  alkaline phosphodiesterase
    ▲——▲  alkaline ϕ-nitrophenylphosphatase
Phosphodiesterase and β-glycerophosphatase

5'-Nucleotidase and 3'-glycerophosphatase (μmoles/min)

(a)

Fraction No.

(b)

Fraction No.

Phosphodiesterase and p-NP phosphatase (μg/min)
activity parallels that of 5'-nucleotidase. Peaks of alkaline p-nitrophenylphatase and alkaline phosphodiesterase activity were also found in the middle fractions of the run (§Fig. III-1).

(ii) By using Sepharose 4-B

Sepharose gels fractionate proteins on the same principle as Sephadex G-200, but are suitable for fractionating the large molecules which are excluded from Sephadex G-200. Whereas Sephadex provides excellent fractionation of globular proteins with molecular weights up to 500000, Sepharose 4-B may be used to separate molecules and particles up to molecular (particle) weights of several millions. The sera of normal and of (UA) tumour-bearing rats were fractionated on such a column in an attempt to determine the molecular weight of the 5'-nucleotidase-carrying material which is excluded from Sephadex G-200. The distributions of proteins and various enzymes after gel filtration of normal serum and the serum of tumour-bearing rats are illustrated in Fig. III-3 and Fig. III-4. The patterns of distribution of proteins for both types of serum seem to be similar. Examination of the distribution of 5'-nucleotidase showed a peak at fraction No. 44. Alkaline β-glycerophosphatase showed a high activity at the same fraction. No peak corresponding to the material excluded from Sephadex G-200 could be detected and there were no significant differences from the pattern obtained with normal serum.

(b) Immunoelectrophoresis techniques

Serum proteins were analysed by crossed immunoelectrophoresis. In the experiments described below, the serum was fractionated by agarose gel electrophoresis in the first dimension, followed by
FIG. III-3  Distribution of protein and various enzymes after fractionating the serum of normal rats on Sepharose 4-B. The column was prepared and eluted in the same way as the column of Sephadex G-200 used in the experiment illustrated in Fig. III-1. Protein in fractions 36-39 is plotted at 1/100 of the scale used for other fractions.

(a) ——— protein

(b) ▲——▲  5'-nucleotidase

□——□  alkaline β-glycerophosphatase
FIG. III-4  Distribution of protein and various enzymes after fractionating the serum of 'UA' tumour-bearing rats on Sepharose 4-B as described in the legend to Fig. III-3. Protein in fractions 36-39 is plotted at 1/100 of the scale used for other fractions.

(a)  protein
(b) ▲—▲  5'-nucleotidase
       □—□  β-glycerophosphatase
electrophoresis into an antibody-containing gel in the second
dimension. The preparation of the agarose plates and the buffer used
have been discussed in Section II-8.

(i) Serum proteins reacting with anti-(rat serum)

Sera of rats carrying (UA) and of rats carrying (WDA) hepatomas
were examined. Fig. III-5 shows the patterns obtained with the sera
of (UA) tumour-bearing and normal rats (the pattern for the serum of
normal rats was obtained by my colleague Dr B. M. Mullock). The
serum of (WDA) hepatoma-bearing rats gives a very similar pattern to
that obtained with the serum of a normal rat. The serum of rats
carrying the (UA) tumour did, however, show some differences. In
normal serum the peak identified as HDL shows greater mobility in
the first dimension than albumin. This peak could not be distinguished
in the serum of rats carrying the (UA) hepatoma. There is also one
peak apparent in the sera of rats carrying the (UA) tumour (marked by
sign '▲') which is not visible in the serum of normal rats. There
are other differences between the two patterns, particularly at the
region marked '□', which are shown clearly in Fig. III-5.

To determine whether HDL was really absent from the serum of
(UA)-carrying rats a lipoprotein fraction was obtained by flotation.
The sera of tumour-bearing and normal rats were mixed with 0.32 g/ml
NaBr and spun for 18 h at 40000 revs/min. The floating fraction was
collected and electrophoresed against anti-(rat serum). The plates
were stained for lipid with Sudan Black-B. Fig. III-6 shows the
patterns obtained which indicate clearly that the HDL is present in
the serum of the tumour-bearing rats.
FIG. III-5 Crossed immunoelectrophoresis of (a) normal rat serum, (b) serum of 'UA' tumour-bearing rat, (c) serum of 'WDA' tumour-bearing rat. Serum samples were diluted 1:10 with 0.15 M NaCl. In the first dimension, an aliquot of about 7 µl of the diluted serum was electrophoresed for about 75 min at 10 v/cm in 1% agarose containing 0.44 M barbitone, pH 8.6. Electrophoresis in the second dimension was against a gel containing 4% anti-(rat serum). These plates are stained with Coomassie Brilliant Blue. The proteins marked are those described in the text.
FIG. III-6  Crossed immunoelectrophoresis of

(a) lipoprotein of normal rat serum
(b) lipoprotein of the serum of 'UA' tumour-bearing rat

These lipoprotein fractions were obtained by flotation from whole serum adjusted to a density of 1.22 g/ml by addition of 0.32 g/ml NaBr and spun for 18 h at 40,000 rev/min. Electrophoresis of undiluted lipoprotein against 4% anti-(rat serum) was carried out as described in Fig. III-5. These plates are stained with Sudan Black-B.
The slight differences between the patterns obtained after crossed immunoelectrophoresis of the serum of normal and tumour-bearing rats against anti-(rat serum) do not mean that there are no other differences between the proteins present in the sera of tumour-bearing rats and the serum of normal rats. As described below, I found a clear difference on crossed immunoelectrophoresis against anti-(hepatoma serum) and anti-(hepatoma plasma membrane) (Section III-3(c)).

(ii) Serum proteins reacting with anti-(plasma membrane)

Crossed immunoelectrophoresis of the sera of (UA) and (WDA) tumour-bearing rats and serum of normal rats against 10% anti-(liver plasma membrane) gave the patterns illustrated in Fig. III-7. Qualitatively, they seem identical but there are quantitative differences in the areas of peaks A and B. Table III-1 shows the results of calculation of the area for each peak.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Type of serum</th>
<th>Area of peak A in mm²</th>
<th>Area of peak B in mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>33.75</td>
<td>35.50</td>
</tr>
<tr>
<td></td>
<td>WDA</td>
<td>49.50</td>
<td>14.25</td>
</tr>
<tr>
<td></td>
<td>UA</td>
<td>49.50</td>
<td>18.00</td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>25.00</td>
<td>27.50</td>
</tr>
<tr>
<td></td>
<td>WDA</td>
<td>39.00</td>
<td>22.00</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>35.00</td>
<td>38.25</td>
</tr>
<tr>
<td></td>
<td>WDA</td>
<td>54.00</td>
<td>16.50</td>
</tr>
<tr>
<td>4</td>
<td>UA</td>
<td>20.25</td>
<td>8.00</td>
</tr>
</tbody>
</table>
FIG. III-7 Crossed immunoelectrophoresis of

(a) normal rat serum
(b) serum of 'UA' tumour-bearing rat (page 68)
(c) serum of 'WDA' tumour-bearing rat

The serum samples were diluted 1:4 with 0.15 M NaCl before electrophoresis. The first dimension was run as described in Fig. III-5. Electrophoresis in the second dimension was against a gel containing 10% anti-(liver plasma membrane). These plates were stained with Coomassie Brilliant Blue. The direction of electrophoresis in the first direction is marked with an arrow. 'A' and 'B' are those which are described in the text.
The results of four separate experiments are given. They show clearly that in the case of serum of normal rats, the area of peak A is less than that of peak B, but that this is reversed in the case of the sera of tumour-bearing rats.

(c) Polyacrylamide gel gradient electrophoresis technique

It is not possible to fractionate such a complicated mixture as the proteins of serum by any single fractionation method. Therefore it is very useful to study such a multicomponent system by 'two-dimensional' separations, in which various components are spread out over a surface. The separation methods used in the two dimensions should exploit different properties of the material. As the separation of serum proteins in agar or agarose gel is dependent on the charges of the molecules, it can be usefully combined with equilibrium electrophoresis in polyacrylamide gel gradients in which the separation is principally dependent on particle size. In practice, a plate of 1% agarose was used, similar to that which was used for immunoelectrophoresis. Slots 16 mm long by 1 mm wide were cut about 2.5 cm from one edge of the plate. Then the sample which had been diluted with an equal volume of 0.9% NaCl was loaded into the slots. After electrophoresis for about 90 min at 10 V/cm the sample slots were filled with agarose and a slab 13 mm wide cut out from each sample lane. This agarose strip was gently pushed edgewise into the loading space between the glass plates at the low density end of a polyacrylamide gradient gel, from which the preservative had been removed by preliminary electrophoresis for 30-45 min at 40V in Tris-borate-EDTA buffer, pH 9.3 [10.12 g Tris + 0.37 g boric acid + 0.96 gm Na₂ EDTA/litre (Leaback, 1976)].
Electrophoresis in the second dimension was carried out for at least 15 h at 40V. The plates were stained for proteins with 0.2% Coomassie Brilliant Blue R in 50% aqueous methanol to which 7 ml/100 ml of glacial acetic acid were added immediately before use, and were destained electrophoretically.

Fig. III-8 shows the results obtained for normal serum (results provided by Dr B. M. Mullock) and the serum of (UA) tumour-bearing rats. The serum of tumour-bearing rats contains distinct proteins not found in the serum of normal animals. Major proteins appear similar in both cases. However, the protein marked (1) in Fig. III-8b, which was also detected in the serum of jaundiced rats (Hinton and Mullock, 1977), is almost certainly the injury macro-globulin (Gordon, 1976) which is essentially absent from the serum of normal rats. Region (2), which contains the β-lipoproteins, shows a quite different appearance in the tumour serum plate from that of normal serum. There are also some bands in the pattern of normal serum which are not visible in the serum of tumour-bearing rats (marked as region (3)).

Serum glycoproteins in polyacrylamide gel gradients

Undiluted serum was used, but otherwise the procedure for two dimensional electrophoresis was as described in the previous paragraph. Finally, the plates were stained for glycoproteins using periodic acid and Schiff's reagent. Fig. III-9 shows the results for the serum of (UA) and (WDA) tumour-bearing rats. The pattern for normal serum obtained by Dr B. M. Mullock is included for comparison. The patterns seem similar and the major proteins contain carbohydrate groups. However, the result obtained for (WDA) tumour-bearing rats
FIG. III-8 Two dimensional electrophoresis of the serum of normal and 'UA' tumour-bearing rat. The first dimension was run in 1% agarose in 0.44 M barbitone buffer, pH 8.6. The second dimension was run in a Gradipore 3-26% polyacrylamide gradient gel in tris-borate-EDTA buffer, pH 9.3. A detailed account of the procedure is given in the text. The samples were diluted 1+1 with 0.15 M NaCl. The direction of electrophoresis in the first dimension is marked in each figure.

(a) normal rat serum
(b) serum of 'UA' tumour-bearing rat

The proteins marked are those described in the text.
FIG. III-9 Pattern of glycoproteins in

(a) normal rat serum
(b) serum of 'UA' tumour-bearing rat
(c) serum of 'WDA' tumour-bearing rat

Two-dimensional electrophoresis was carried out as described in Fig. III-8, except that the serum was not diluted prior to use. These plates were stained for glycoprotein using periodic acid and Schiff's reagent. The direction of electrophoresis in the first dimension is marked on each figure.
shows slight differences from that of normal serum at the region (marked (4)) in Fig. III-9c. Since the protein in this region moves more slowly than albumin, the protein may be α-fetoprotein (AFP), which has a molecular weight 75100 higher than albumin (Lowing, 1977). Alternatively, it could be a specific protein in the serum of tumour-bearing rats as described below (Section III-3c).

III-2 ISOLATION OF PLASMA MEMBRANE FROM NORMAL LIVER AND HEPATOMA

(a) Electron microscope studies of the plasma membrane of hepatoma cells

Sections from two different hepatomas were examined by electron microscopy (using a JEM electron microscope). The procedure for preparing these sections was described in Chapter II-6. Fig. III-10 shows a section taken from the slow growing 'WDA' tumour-carrying rat. The figure shows clearly the cell plasma membrane (marked PM). When following the plasma membrane around the cells one finds a region whose structure, between cells, resembles that of the bile canaliculus in liver cells. This is shown in more detail in Fig. III-11(a). Furthermore, in other regions of the tumour cell, the appearance of the plasma membrane resembles the sinusoidal face region of the liver cell (Fig. III-11(b)).

The appearance of cells from the fast-growing 'UA' hepatoma (Fig. III-12) was rather different. This figure shows that the plasma membrane of the 'UA' tumour has less structural organisation than that of the 'WDA' tumour. However, junctional complexes between adjacent cells are seen in Fig. III-13. On further examination of this and
FIG. III-10  Electron micrograph of a section from the 'WDA' hepatoma. The section was fixed with 1% glutaraldehyde and in 2% osmic acid. After embedding, the section was counterstained with alkaline lead citrate and uranyl acetate. The regions marked by the arrows are shown in the next figure at higher magnification.

Magnification $\times$ 8640
FIG. III-11

(a) A detail of the section shown in Fig. III-10 which shows a region whose structure, between cells, resembles that of the bile canaliculus in liver cells.

Magnification × 20,000

(b) A details of the section shown in Fig. III-10 which shows a region whose structure resembles the sinusoidal face region of the liver cell.

Magnification × 22,000
FIG. III-12 Electron micrograph of a section from the 'UA' hepatoma. The section was treated as described in Fig. III-10. The region marked by the arrow is shown in the next figure at higher magnification.

Magnification × 7100
FIG. III-13  A detail of the section shown in Fig. III-12. This region shows a junctional complex between adjacent cells.

Magnification × 12,800
many other sections we could not find a structure resembling that of bile canaliculi as in liver cells.

(b) Isolation of large sheet fragments of plasma membrane

(i) Normal liver

As mentioned in the Introduction, many investigators have shown that, after homogenisation of liver, the plasma membrane breaks into large sheets which sediment with nuclear fraction, and small vesicles recovered in the microsomal fraction. Methods for the purification of plasma membrane sheets from nuclear fraction have been reported from this laboratory (Hinton et al., 1970); Issa, 1976). Since the red blood cells aggregate with the plasma membrane fragments, the liver was perfused with 0.25 M sucrose to remove the red blood cells. However, in my study the liver was not perfused but the plasma membrane fragments were separated from the red blood cells by flotation. In brief, the livers were homogenised in 0.25 M sucrose containing 5 mM tris-HCl pH 8. After filtration through a coarse sieve, the density of the homogenate was adjusted to density 1.18 g/ml (ref. index = 1.40) and after overlaying with 0.25 M sucrose, spun in an (8 x 50) ml angle-head rotor for 30 min at 12000 revs/min. The interface layer was removed with a bent pipette and diluted four times with 0.25 M sucrose then spun again and a sample was taken as a reference. The pellet was washed twice by resuspension in 0.25 M sucrose and pelleting using a M.S.E. Minor bench centrifuge. Each centrifugation was for 15 min, the first being at setting No. 8 and the second at setting No. 5. Finally, the pellet was suspended in 30 ml of 0.25 M sucrose. The enzymes assayed were: 5'-nucleotidase, glucose-6-phosphatase, acid phosphatase and succinate dehydrogenase, which were respectively used as markers for
plasma membrane, endoplasmic reticulum, lysosomes and mitochondria. Table III-2 shows the results of purification and yield of these various marker enzymes.

The results showed that a reasonable purification of plasma membrane fragments as indicated by 5'-nucleotidase, was achieved by the procedure described above. But this plasma membrane fraction still contained as contaminants significant amounts of endoplasmic reticulum, mitochondria and a very low amount of lysosomes as judged by their marker enzymes, glucose-6-phosphatase, succinate dehydrogenase and acid phosphatase respectively. The comparison of these results with those obtained by Issa (1976) is discussed in Chapter IV.

(ii) Hepatoma

A method for the isolation and purification of plasma membrane from a slow-growing hepatoma was developed in this laboratory by Prospero (1975) who used centrifugal techniques employing exponential gradients (details of the gradient are described in Section II-5(a)). As mentioned in Chapter II, Materials and Methods, two sublines of tumour were used in the present study, the first of which is fast growing with a generation time between 2-3 weeks ('UA' tumour) and the second is slower growing with a generation time of about 6 weeks ('WDA' tumour). Attempts were made to separate plasma membrane fragments from both, using the techniques of Prospero (1975). The floated fraction, obtained from the whole hepatoma homogenate after adjusting to a density of 1.18 g /ml and centrifuging for 50 min at 133,000 xg, was loaded into the HS zonal rotor and centrifuged for 18 min at 9,000 revs/min. Figs. III-14 and III-15 show the
TABLE III-2  Purification and yield of various marker enzymes during preparation of plasma membrane fraction by centrifugation the whole liver homogenate for 30 min at 12,000 rev/min in 8 x 50 ml rotor. (The results of two separate experiments are given.)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>% Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μ moles/ mg protein/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.069 ; 0.053</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Fraction floating at</td>
<td>0.127 ; 0.076</td>
<td>37.59 ; 29.44</td>
<td>1.82 ; 1.41</td>
</tr>
<tr>
<td>density = 1.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction after</td>
<td>0.902 ; 0.674</td>
<td>26.1 ; 12.58</td>
<td>13 ; 12.57</td>
</tr>
<tr>
<td>pelleting and washing</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Glucose-6-phosphatase

| Homogenate               | 0.082 ; 0.053     | 100     | 1            |
| Fraction floating at     | 0.045 ; 0.042     | 11.409 ; 16.38 | 0.55 ; 0.79 |
| density = 1.18           |                   |         |              |
| Fraction after           | 0.026 ; 0         | 0.648 ; 0 | 0.32 ; 0    |
| pelleting and washing    |                   |         |              |

Acid phosphatase

| Homogenate               | 0.049 ; 0.046     | 100     | 1            |
| Fraction floating at     | 0.045 ; 0.051     | 18.89 ; 13.76 | 0.919 ; 0.67 |
| density = 1.18           |                   |         |              |
| Fraction after           | 0.008 ; 0         | 0.33 ; 0 | 0.164 ; 0   |
| pelleting and washing    |                   |         |              |

Succinate dehydrogenase

| Homogenate               | 0.047 ; 0.034     | 100     | 1            |
| Fraction floating at     | 0.016 ; 0.032     | 7.16 ; 19.53 | 0.347 ; 0.947 |
| density = 1.18           |                   |         |              |
| Fraction after           | 0.027 ; 0.0058    | 0.59 ; 0.26 | 0.589 ; 0.17 |
| pelleting and washing    |                   |         |              |
FIG. III-14  Distribution of protein and marker enzymes after centrifugation of a fraction isolated from a 'UA' hepatoma homogenate by flotation at density 1.18. Centrifugation was for 18 min at 9000 revs/min in the HS zonal rotor.

(a) — — — — density
   ○——○  5'-nucleotidase
   ■——■  glucose 6-phosphatase

(b) — — — — protein
   ●——●  alkaline p-nitrophenylphosphatase
   △——△  alkaline β-glycerophosphatase

(c) ▼ ▼ alkaline phosphodiesterase
   +——+  acid β-glycerophosphatase
   □——□  succinate dehydrogenase
FIG. III-15 Distribution of protein and marker enzymes after centrifugation of a fraction isolated from a 'WDA' hepatoma homogenate by flotation at density 1.18. Centrifugation was for 18 min at 9000 revs/min in the HS zonal rotor.

(a)  [Diagram] density
     ○ - ○  5'-nucleotidase
     ■ - ■  glucose 6-phosphatase

(b)  [Diagram] protein
     • - •  alkaline P-nitrophenylphosphatase
     △ - △  alkaline β-glycerophosphatase

(c)  [Diagram] alkaline phosphodiesterase
     ▲ - ▲  acid β-glycerophosphatase
distribution of protein and various marker enzymes after fractionating material floated from homogenates of the 'UA' and 'WDA' tumours.

The early part of the gradient (fractions 3-7) contains soluble proteins and small particulate components which have not sedimented away from the sample region. However, a clear zone of plasma membrane fragments, indicated by 5'-nucleotidase activity is found at a density of between 1.12-1.15 g/ml. Analysis of marker enzymes showed that the plasma membrane zone in the central part of the gradient is only slightly contaminated by endoplasmic reticulum fragments and lysosomes. There was a significant contamination by mitochondria in the case of preparing large sheets of plasma membrane from the 'UA' tumour while there were virtually no mitochondria in preparing large sheets of plasma membrane from 'WDA' tumour, as judged by the lack of succinate dehydrogenase activity.

Tables III-3 and III-4 show the purification and yield of various marker enzymes for both tumours. The Figures and Tables also show the distribution of other enzymes such as alkaline phosphodiesterase and alkaline $\beta$-glycerophosphatase, which appear to be almost entirely localised in the plasma membrane fragments.

(c) Separation of plasma membrane vesicles

The microsomal fraction was obtained by centrifuging at 40,000 revs/min for 1 h the supernatant obtained from the hepatoma homogenate centrifugation at 10,000 revs/min for 15 min. The pellet of microsomes was suspended in 2 M sucrose containing as a buffer 5 mM tris-HCl pH 8. After a sample had been taken out as a reference, the rest of the fraction (22 ml) was injected into the edge of the
TABLE III-3  Purification and yield of various marker enzymes during preparation of plasma membrane fraction by centrifugation of the floated material from the whole (UA) hepatoma homogenate for 18 min at 9,000 rev/min in HS zonal rotor. The results are given as means ± standard error. Unless otherwise indicated the results are averaged from two experiments.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>% Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.186 ± 0.0103</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>1.33 ± 0.121</td>
<td>9.6 ± 0.625</td>
<td>6.77</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>1.77 ± 0.588</td>
<td>1.32 ± 0.632</td>
<td>9.95</td>
</tr>
<tr>
<td>Glucose-6-phosphatase (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.0067 ± 0.0023</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>0.0167 ± 0.0032</td>
<td>3.305 ± 1.635</td>
<td>2.27</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.01 ± 0.005</td>
<td>0.376 ± 0.162</td>
<td>1.36</td>
</tr>
<tr>
<td>Acid phosphatase (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.025 ± 0.0006</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>0.078 ± 0.012</td>
<td>3.032 ± 1.536</td>
<td>3.03</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.033 ± 0.016</td>
<td>0.268 ± 0.146</td>
<td>1.29</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.0108</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>0.0048</td>
<td>0.84</td>
<td>0.584</td>
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<tr>
<td>Zonal plasma-membrane</td>
<td>0.0132</td>
<td>0.27</td>
<td>1.443</td>
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Cont.
<table>
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<th>TABLE III-3 (Cont)</th>
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<tr>
<td><strong>Alkaline β-glycerophosphatase (3)</strong></td>
</tr>
<tr>
<td>Homogenate</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>µg/ mg protein/min</th>
<th>% Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkaline phosphodiesterase (3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>2.04 ± 0.336</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>12.04 ± 1.87</td>
<td>9.40 ± 0.59</td>
<td>10.33</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>22.92 ± 9.67</td>
<td>1.74 ± 0.368</td>
<td>10.67</td>
</tr>
</tbody>
</table>

<p>| <strong>Alkaline η-nitrophenylphosphatase (3)</strong> |
| Homogenate        | 2.41 ± 0.223      | 100    | 1           |
| Floated material loaded into the zonal rotor | 14.81 ± 1.108 | 9.55 ± 0.59 | 6.47 |
| Zonal plasma-membrane | 27.44 ± 13.45 | 1.75 ± 0.527 | 10.89 |</p>
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity μ moles/mg protein/min</th>
<th>% Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.053</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material</td>
<td>0.609</td>
<td>12.43</td>
<td>11.49</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.576</td>
<td>4.25</td>
<td>10.86</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.006</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material</td>
<td>0.02</td>
<td>3.4</td>
<td>3.33</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.014</td>
<td>0.9</td>
<td>2.46</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.024</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material</td>
<td>0.097</td>
<td>4.27</td>
<td>3.89</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.091</td>
<td>1.44</td>
<td>3.65</td>
</tr>
<tr>
<td>Alkaline β-glycerophosphatase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.015</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material</td>
<td>0.316</td>
<td>22.71</td>
<td>21.06</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.389</td>
<td>10.11</td>
<td>25.93</td>
</tr>
</tbody>
</table>

(Cont.)
<table>
<thead>
<tr>
<th></th>
<th>Specific activity $\mu$g/\text{mg protein/min}$</th>
<th>% Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkaline $\rho$-nitrophenylphosphatase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>5.92</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>78.31</td>
<td>0.14</td>
<td>13.22</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>108.42</td>
<td>7.22</td>
<td>18.31</td>
</tr>
<tr>
<td><strong>Alkaline phosphodiesterase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>2.32</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Floated material loaded into the zonal rotor</td>
<td>25.28</td>
<td>11.85</td>
<td>10.89</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>32.28</td>
<td>5.48</td>
<td>13.91</td>
</tr>
</tbody>
</table>
B-XIV zonal rotor, under the linear gradient. The rotor was then filled completely with 2 M sucrose as a cushion and was accelerated to 44,000 revs/min. After centrifugation for about 16 h the rotor was decelerated to the loading speed and the contents displaced. The distribution of marker enzymes and protein after fractionation of microsomes from tumours 'UA' and 'WDA' are shown in Figs. III-16 and III-17. The first sharp peak at density range between 1.11-1.14 represents the plasma membrane fragments as judged by the 5'-nucleotidase pattern. The bulk of the activity of glucose-6-phosphatase is found at the second peak, which indicates that the endoplasmic reticulum fragments are located at the density range 1.17-1.20. The lysosomal marker acid phosphatase shows a pattern similar to the 5'-nucleotidase and therefore indicates that the plasma membrane region is contaminated with lysosome fragments.

From the distribution of marker enzymes one can conclude that a reasonably pure sample of a plasma membrane fragment can be obtained by pooling fractions (4-9). These fractions are only slightly contaminated by other subcellular organelles such as endoplasmic reticulum, lysosomes and mitochondria (Figs. III-16 and III-17). The plasma membrane fragments used to raise the anti-(tumour plasma membrane) antiserum were prepared in this way. The pooled fractions were diluted with an equal volume of distilled water and the vesicles collected by centrifugation for 90 min at 40,000 revs/min. The pellet was then washed twice by resuspension in 0.15 M NaCl and recentrifuged for 90 min at 40,000 revs/min.

The purifications and yields of various marker enzymes are shown in Tables III-5 and III-6 for both tumours. It must also be mentioned
FIG. III-16 Distribution of protein and marker enzymes after flotation for 16 h at 44,000 revs/min in a B-XIV zonal rotor of the microsomal fraction separated from a 'UA' hepatoma homogenate by centrifugation for 60 min at 40,000 revs/min of the supernatant remaining after large particles had been removed by centrifugation for 15 min at 10,000 revs/min.

The microsomal fraction was resuspended in 25 ml of 2M-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) density
   ●●●● 5'-nucleotidase
   □□□□ glucose 6-phosphatase

(b) protein
   ++++++ AT-pase
   ●●●● alkaline γ-nitrophenylphosphatase

(c) succinate dehydrogenase
   □□□□ acid β-glycerophosphatase
   ▼▼▼▼ alkaline phosphodiesterase
   △△△△ alkaline β-glycerophosphatase
alkaline β-glycerophosphatase
(△—△)
acid β-glycerophosphatase
(○—○)
succinate dehydrogenase
(●—●)

adenosinetriphosphatase
(+—+—+)
alkaline p-nitrophenyl phosphate
(●—●)

5'-nucleotidase
(○—○)
glucose 6-phosphatase
(■—■)

% of total activity in each fraction

alkaline phosphodiesterase
(▼—▼)

Protein (— — —)

Density (———)
FIG. III-17 Distribution of protein and marker enzymes after flotation for 16 h at 44,000 revs/min of the microsomal fraction of a 'WDA' hepatoma homogenate. Experimental conditions were the same as in the experiment illustrated in Fig. III-16.

(a) ——— density
     ○———○ 5'-nucleotidase
     ■——■ glucose 6-phosphatase
     ——— protein

(b) △——△ alkaline β-glycerophosphatase
    •——• alkaline p-nitrophenylphosphatase

(c) ▼——▼ alkaline phosphodiesterase
    □——□ acid β-glycerophosphatase
TABLE III-5  Purification and yield of various marker enzymes during preparation of plasma membrane fraction by centrifugation of microsomal fraction of (UA) hepatoma for 16 h at 44,000 revs/min in B-XIV zonal rotor. The results are given as means ± standard error. Unless otherwise indicated the results are averaged from two experiments.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (μ moles/mg protein/min)</th>
<th>% Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.191 ± 0.0155</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.338 ± 0.0099</td>
<td>~18.47 ± 0.465</td>
<td>1.78</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>2.128 ± 0.365</td>
<td>8.84 ± 1.45</td>
<td>11.10</td>
</tr>
<tr>
<td>Glucose-6-phosphatase (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.0062 ± 0.0004</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.0094 ± 0.001</td>
<td>15.47 ± 1.77</td>
<td>1.52</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.455 ± 0.0007</td>
<td>1.824 ± 0.571</td>
<td>2.10</td>
</tr>
<tr>
<td>Acid phosphatase (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.028 ± 0.0023</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.053 ± 0.0035</td>
<td>19.32 ± 0.861</td>
<td>1.86</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.171 ± 0.0412</td>
<td>4.74 ± 1.072</td>
<td>5.84</td>
</tr>
<tr>
<td>Alkaline β-glycerophosphatase (6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.0082 ± 0.001</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.104 ± 0.001</td>
<td>13.2 ± 0.47</td>
<td>1.28</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.0508 ± 0.0095</td>
<td>4.88 ± 0.89</td>
<td>6.25</td>
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</tbody>
</table>
TABLE III-5 (Cont)

<table>
<thead>
<tr>
<th></th>
<th>Succinate dehydrogenase</th>
<th>Adenosine triphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>μg g / mg protein/min</td>
<td>% Yield</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Purification</td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.0136</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.0052</td>
<td>4.05</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.0044</td>
<td>0.461</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.386</td>
</tr>
</tbody>
</table>

|                          |                          | Alkaline P-nitrophenylphosphatase(6) |
|                          |                          |                             |
|                          | Specific activity       | % Yield                  |
|                          | μg g / mg protein/min   | Purification              |
| Homogenate               | 1.72 ± 0.643            | 100                       |
|                          |                         | 1                         |
| Material loaded into the zonal rotor | 3.58 ± 1.21 | 25.16 ± 5.02 | 2.48 |
| Zonal plasma-membrane   | 23.09 ± 11.81           | 11.7 ± 4.40               |
|                          |                         | 14.27                     |

|                          |                          | Alkaline phosphodiesterase (6) |
|                          |                          |                             |
|                          | Specific activity       | % Yield                  |
|                          | μg g / mg protein/min   | Purification              |
| Homogenate               | 1.44 ± 0.182            | 100                       |
|                          |                         | 1                         |
| Material loaded into the zonal rotor | 3.32 ± 0.292 | 24.30 ± 2.45 | 2.56 |
| Zonal plasma-membrane   | 17.14 ± 4.225           | 9.06 ± 0.917              |
|                          |                         | 12.06                     |
TABLE III-6  Purification and yield of various marker enzymes during preparation of plasma membrane by centrifugation of microsomal fraction of (WDA) hepatome for 16 h at 44,000 revs/min in B-XIV zonal rotor. (The results given are for two experiments.)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (μ mole/mg protein/min)</th>
<th>% Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5'-Nucleotidase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.081</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.24</td>
<td>19.30</td>
<td>2.98</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.846</td>
<td>13.02</td>
<td>10.62</td>
</tr>
<tr>
<td><strong>Glucose-6-phosphatase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.0073</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.0115</td>
<td>9.90</td>
<td>1.51</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.024</td>
<td>4.25</td>
<td>3.24</td>
</tr>
<tr>
<td><strong>Acid phosphatase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.032</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.115</td>
<td>23.98</td>
<td>3.93</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.149</td>
<td>5.80</td>
<td>4.85</td>
</tr>
<tr>
<td><strong>Alkaline β-glycerophosphatase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.045</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>0.181</td>
<td>25.62</td>
<td>4.09</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>0.484</td>
<td>12.75</td>
<td>10.88</td>
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</table>

Cont.
<table>
<thead>
<tr>
<th></th>
<th>Specific activity</th>
<th>% Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ mg protein/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Alkaline p-nitrophenylphosphatase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>10.43</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>43.72</td>
<td>26.09</td>
<td>4.09</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>116.64</td>
<td>14.01</td>
<td>11.03</td>
</tr>
<tr>
<td><strong>Alkaline phosphodiesterase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>2.61</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Material loaded into the zonal rotor</td>
<td>11.69</td>
<td>28.24</td>
<td>4.44</td>
</tr>
<tr>
<td>Zonal plasma-membrane</td>
<td>26.93</td>
<td>12.46</td>
<td>10.24</td>
</tr>
</tbody>
</table>
that this region shows activities for other plasma membrane enzymes such as ATPase, alkaline phosphatase assayed with \( \text{p-nitrophenyl phosphate} \) as substrate, and alkaline phosphodiesterase. This observation means that those enzymes exist in the same site of plasma membrane fragments. The comparison between two sets of results for 'UA' and 'WDA' tumours, obtained by using the above procedure, is discussed in the next chapter.

(d) **Fractionation of membrane proteins on polyacrylamide gels**

Fractions from an experiment similar to that illustrated in Fig. III-16 were pooled in groups of four. The pooled fractions were diluted with water and particulate material collected by centrifugation and washed by resuspension with 0.15 M NaCl. Samples from these suspensions were treated with SDS and mercaptoethanol as described in Section II-8, and fractionated on polyacrylamide gels. The method for preparing the gels and the buffers which were used has been described above. The patterns obtained are shown in Fig. III-18. The first fractions, Fig. III-18(a) (fractions 1-4) contain very little protein while the others contain quite a lot of material as shown by the patterns of the bands. The bands numbered 1 to 5 are found in all fractions. Comparison with the distribution of enzymes (Fig. III-16) suggests that these proteins are associated with the endoplasmic reticulum. However, Fig. III-18(b) which represents the fractions rich in plasma membrane fragments, also shows additional bands, presumably due to plasma membrane components. These results give further evidence that the plasma membrane fragments are contaminated by other organelles such as endoplasmic reticulum.
FIG. III-18 Polyacrylamide-gel electrophoresis in 0.05 M tris - 0.4 M glycine buffer, pH 8.3 of the proteins of microsomal subfractions separated from the microsomal fraction of a 'UA' hepatoma homogenate as illustrated in Fig. III-16.

The pooled fractions were diluted with distilled water and particulate material collected by centrifugation for 90 min at 40,000 rev/min and washed by resuspension with 0.15 M NaCl and repelleting.

Electrophoresis was performed for 3 h at room temperature using 5 mA/tubes. The gels are stained with Coomassie Brilliant Blue-G. The bands marked 1 to 5 are those discussed in the text.

(a) Pool of fractions 1 to 4
(b) Pool of fractions 5 to 9
(c) Pool of fractions 10 to 13
(d) Pool of fractions 27 to 30
Ill-3 COMPARISON OF PROTEINS IN THE SERUM OF NORMAL AND TUMOUR-BEARING RATS

(a) Proteins reacting with antisera prepared against the serum of normal and 'UA' tumour-bearing rats

Crossed immunoelectrophoresis of serum from both 'UA' and 'WDA' tumour-bearing rats against an antiserum prepared against the serum of 'UA' tumour-bearing rats (anti 'UA' serum) gave a complex pattern (Fig. III-19(a) and III-19(b)). The patterns seem identical, at least as regards the main proteins. However, by using an intermediate gel technique it was possible to demonstrate that the serum of 'UA' and 'WDA' tumour-bearing rats each show a specific protein (Fig. III-20(b) and Fig. III-20(c)), neither of which is present in the serum of normal rats (Fig. III-20(a)). The two tumour specific proteins differ in their mobility in the first dimension. In the case of the serum of 'UA' tumour-bearing rats, this specific protein moves more slowly than albumin, while its counterpart in the serum of 'WDA' migrates as a prealbumin. The serum of rats carrying the 'UA' hepatoma was also tested against an antiserum prepared against the serum of laparotomised animals (Fig. III-20(d)). Experiments by other workers in this laboratory had shown that this antiserum contains antibodies reacting with some 'injury' proteins. However, the pattern obtained with the serum of tumour-bearing rats is indistinguishable from that obtained using normal serum (§Fig. III-20(a)).

(b) Proteins reacting with anti-(liver plasma membrane) and anti-('UA' hepatoma plasma membrane)

Sera from 'UA' and 'WDA' tumour-bearing rats and normal serum
FIG. III-19 Crossed immunoelectrophoresis of

(a) serum of 'UA' tumour-bearing rat
(b) serum of 'WDA' tumour-bearing rat

The first dimension was run as described in Fig. III-5. Electrophoresis in the second dimension was against a gel containing 5% anti-(serum of 'UA' tumour-bearing rat). The plates are stained with Coomassie Brilliant Blue.
FIG. III-20  Crossed immunoelectrophoresis of

(a) normal rat serum
(b) and (d) serum of 'UA' tumour-bearing rat
(c) serum of 'WDA' tumour-bearing rat

The first dimension was run as described in Fig. III-5. The second dimension was run for 18 h at 2 V/cm against a split antibody-containing gel made up as follows:

(a), (b) and (c) 10% anti-(rat serum) in the lower part and 10% anti-(serum of 'UA' tumour-bearing rat) in the upper part.
(d) 10% anti-(rat serum) in the lower part and 10% anti-(serum of laparotomised rat) in the upper part.

The plates were stained with Coomassie Brilliant Blue. The proteins marked are those described in the text.
were examined by crossed immunoelectrophoresis against anti-('UA' hepatoma plasma membrane) prepared as described in Section II-9 (Figs.III-21(a-c)). The antiserum reacted strongly with a number of major serum proteins. Three proteins showed interesting changes between normal serum and the serum of tumour-bearing rats. These are marked (A), (B) and (C) in the Figures. Protein (A) appears as a double peak in all sera, but the proportion of the more rapidly moving component is greater in the serum of tumour-bearing rats. Examination of the patterns shows a specific protein, (B), is present in the sera of tumour-bearing rats but not in normal serum (Figs.III-21(b), (c)). Finally an extra protein, (C), is present in the sera of rats carrying the 'UA' hepatoma but absent from the serum of rats carrying the 'WDA' tumour and normal serum. Further experiments showed that protein (B) has antigenic determinants in common with some protein of normal serum (Figs.III-22(a), (b)), but that protein (C) is immunologically unrelated to normal serum protein.

Figs. III-23 (a), (b) show the patterns obtained of the serum of 'UA' and 'WDA' tumour-bearing rats after crossed immunoelectrophoresis against a split antibody gel containing 10% anti-(liver plasma membrane) in the lower gel, and 10% anti-('UA' hepatoma plasma membranes) in the upper gel. These figures show that neither proteins (B) nor (C) react with anti-(liver plasma membrane).
FIG. III-21 Crossed immunoelectrophoresis of

(a) normal rat serum
(b) serum of 'UA' tumour-bearing rat
(c) serum of 'WDA' tumour-bearing rat.

The first dimension was run as described in Fig. III-5.

(a) Electrophoresis in the second dimension was against a split antibody-containing gel as 10% anti-('UA' hepatoma plasma membrane) in the lower part and 10% anti-(rat serum) in the upper part.

(b) and (c) Electrophoresis in the second dimension was against a gel containing 10% anti-('UA' hepatoma plasma membrane).

The plates are stained with Coomassie Brilliant Blue. 'A', 'B' and 'C' indicate the three proteins which are discussed in the text.
FIG. III-22 Crossed immunoelectrophoresis of

(a) normal rat serum
(b) serum of 'UA' tumour-bearing rat

The first dimension was run as described in Fig. III-5. Electrophoresis in the second dimension was against a split antibody-containing gel with 10% anti-(rat serum) in the lower part and 10% anti-('UA' hepatoma plasma membrane) in the upper part. The plates are stained with Coomassie Brilliant Blue.
FIG. III-23 Crossed immunoelectrophoresis of
(a) serum of 'UA' tumour-bearing rat
(b) serum of 'WDA' tumour-bearing rat

The first dimension was run as described in Fig. III-5. Electrophoresis in the second dimension was against a split antibody-containing gel as 10% anti-(liver plasma membrane) in the lower part and 10% anti-('UA' hepatoma plasma membrane) in the upper part. The plates are stained with Coomassie Brilliant Blue.
(a) Studies on 5'-nucleotidase in serum and the plasma membrane of normal liver and hepatoma

(i) Comparison of the properties of liver plasma membrane and 5'-nucleotidase

The pH-dependence of 5'-nucleotidase in serum and plasma membrane fragments was examined using a variety of buffers. Figs. III-24(a), (b) show the patterns which were obtained. Fig. III-24(a) shows that the pH optimum of plasma membrane 5'-nucleotidase depends on the buffer employed. With tris buffer the pH optimum of plasma membrane 5'-nucleotidase was between 7.3-7.5. Higher activities were obtained with glycine and glycylglycine buffer and in these buffers the pH optimum was around 9.0. The results obtained with bicarbonate buffer were consistent with a similar pH optimum but the absolute activities were much lower than those obtained with glycine or glycylglycine at the same pH.

Similar studies were carried out on the 5'-nucleotidase in normal rat serum. Five different buffers were employed. The enzyme activity in normal serum showed a much more marked dependence upon the pH than the enzyme in liver plasma membrane. A change of 0.5 pH units produces a several-fold increase in activity. Fig. III-24(b) shows the pH curves obtained with each buffer. Dimethylglutarate appeared to inhibit the enzyme. With tris buffer the optimum pH was around 8.5. Bicarbonate and glycylglycine gave almost the same activity as tris but at a pH optimum of 9.0. Glycine appeared to inhibit the enzyme but the results are consistent with an optimum pH of around 8.5.
FIG. III-24 Activity of 5′-nucleotidase in

(a) liver plasma membrane fragments
(b) normal rat serum

as a function of pH. The buffers employed were as follows:

▼▼ tris
▲▲ glycine
○○ bicarbonate
■■■ glycylglycine
●● dimethylglutarate
The relationship between hepatoma and liver 5'-nucleotidase

Hepatoma plasma membranes are rich in 5'-nucleotidase. The relationship of this enzyme to the 5'-nucleotidase of liver plasma membrane has been examined by using the Ouchterlony double diffusion technique. In brief, a sample of hepatoma plasma membrane fragments was extracted by treatment with an equal volume of a detergent mixture (0.5% Lubrol and 1% deoxycholate) for about 2 h at 0°C. The mixture was centrifuged at 50,000 revs/min for \( \frac{1}{2} \) h to remove insolubilised proteins. Plates of 1.5% agar in 0.3 M phosphate buffer pH 7.4 were prepared and the wells cut using a home-made cutter. The sample at three different concentrations was loaded into some of the wells while others were filled with anti-liver plasma membrane, anti-cytosol and anti-liver microsomes as shown in Fig. III-25. The plates were left for two days. Unprecipitated proteins were removed by washing with several changes of 25% saline for two days. The plates were then washed with distilled water to remove salt. One of the plates was incubated at 37°C for about 2 h with 30 ml of a staining mixture (50 mM DMG pH 7.5, 2 mM Pb(NO₃)₂, 5 mM Mg(NO₃)₂ and 50 mM UMP) while the other plate, used as a blank, was incubated for the same period of time with the same mixture but without 50 mM UMP. The plates were rinsed with distilled water and then immersed in a 0.2% solution of yellow ammonium sulphide for a few minutes. Fig. III-25 shows the pattern obtained. There are clear lines between the wells which contain the samples of hepatoma plasma membrane and those containing anti-liver plasma membrane or anti-liver microsomes. This indicates that the 5'-nucleotidase of hepatoma plasma membrane is identical to that of liver plasma membrane.
FIG. III-25 Reaction of 5'-nucleotidase in extracts of 'UA' hepatoma homogenate membranes with a variety of antisera. The sample was extracted by treatment with an equal volume of a detergent mixture (0.5% lubrol and 1% deoxycholate) for about 2 h at 0°C, and centrifugation for 30 min at 50,000 revs/min to remove insolubilised proteins. Three different concentrations of the sample were used against anti-(liver plasma membrane), anti-(liver cytosol) and anti-(liver microsomes). The diffusion and precipitation were allowed to proceed for 48 h at room temperature. The unprecipitated proteins were washed off by soaking in 2.5% NaCl for 2 days followed by distilled water. The plates are stained for 5'-nucleotidase as described in the text.

(a) The incubation mixture contained (50 mM UMP)
(b) The plate contained no UMP
The pH-dependence of the p-nitrophenylphosphatase in plasma membrane fragments was examined using five different buffers. The results are shown in Fig. III-26(a). The results with tris and glycylglycine show a pattern which suggests an optimum at a pH of around 7.5. There would, however, appear to be a second pH optimum, shown most clearly with bicarbonate buffer at a pH of around 10.0. The results with glycine buffer are difficult to interpret. The results obtained with dimethylglutarate show that there is considerable activity at an acid pH. Earlier results in our laboratory (Hinton, 1970) show that this is not due simply to lysosomal acid phosphatase.

The pH-dependence of alkaline p-nitrophenylphosphatase in rat serum was determined using four different buffers. The results are shown in Fig. III-26(b). All buffers give an optimum pH of around 9.5 although the activity obtained with glycine buffer was much lower than that obtained with the other buffer employed in this study. It should be noted that similarity of the changes with pH of serum alkaline p-nitrophenylphosphatase and 5'-nucleotidase suggests that the two activities are due to a single enzyme. This enzyme is activated by Mg\(^{++}\) ions (Fig. III-27) and has a \(k_m\) of 3 mM when assayed at pH 9.7 with p-nitrophenylphosphate as substrate (Fig. III-28).
FIG. III-26 Activity of alkaline \( \rho \)-nitrophenylphosphatase in
(a) liver plasma membrane fragments
(b) normal rat serum
as a function of pH. The buffers employed were as follows:

- •••• dimethylglutarate
- ▼▼ tris
- ▲▲ glycine
- ○○ bicarbonate
- ■■ glycylglycine
FIG. III-27 Activity of alkaline $p$-nitrophenylphosphatase in the serum of a normal rat as a function of the concentration of MgCl$_2$ or EDTA in the assay medium. The assays were carried out in a bicarbonate buffer, pH 9.5.
FIG. III-28  Estimation of Km of alkaline p-nitrophenylphosphatase by replotting the data from Fig. II-2 according to Lineweave-Burk.
CHAPTER FOUR

DISCUSSION
(a) Isolation of large sheet fragments of plasma membrane

(i) Normal liver

As discussed in Section III-2(b), I attempted to separate plasma membrane from normal rat liver homogenised in 0.25 M sucrose without prior perfusion. The normal methods used in our laboratory (e.g. Hinton et al., 1970; Issa, 1976) employ perfused liver. The reason for using non-perfused normal rat liver was to determine whether there was loss of the plasma membrane fragments as compared with the method using perfused rat liver. This is relevant to the isolation of sheets of hepatoma plasma membrane because the lack of a main blood vessel makes it difficult to perfuse tumours. The marker enzymes, 5'-nucleotidase, glucose 6-phosphatase, acid phosphatase and succinate dehydrogenase were respectively used as indicators of the plasma membrane, endoplasmic reticulum, lysosomes and mitochondria.

Comparison with the results of Issa (1976), who used a centrifugation in an A-XII zonal rotor to fractionate the material floated from the whole liver homogenate, suggests that the separation with unperfused liver is comparable to that obtained using perfused normal rat liver. Assay of the enzymes G-6-pase, acid phosphatase and succinate dehydrogenase indicates that the fraction prepared by flotation from the whole homogenate contains considerable amounts of other organelles such as endoplasmic reticulum, lysosomes and mitochondria as well as plasma membrane. In my experiments, the plasma membrane fragments were partially separated from the contaminating organelles by twice resuspending in 0.25 M sucrose and pelleting in
the bench centrifuge. Issa (1976) purified the plasma membrane fragments by centrifugation in an A-XII zonal rotor and hence obtained a greater purification in the final step. Thus, it would seem that, provided that plasma membranes and erythrocytes are separated by flotation, it is possible to prepare plasma membranes from the livers of rats homogenised in 0.25 M sucrose without prior perfusion of the liver. It would, therefore, appear valid to use a similar approach in isolating plasma membrane from hepatoma.

(ii) Hepatoma

As mentioned in 'Aims of this study', a method for isolating purified plasma membrane sheets from the slow growing 'WDA' tumour had been developed in this laboratory prior to my studies (Prospero, 1975). I have used the same techniques for both the slow growing 'WDA' tumour and the faster growing 'UA' tumour to examine whether that method is still applicable following progression of the 'WDA' tumour and whether it is useful for both tumours. The patterns obtained for the distributions of protein and marker enzymes during preparation of plasma membrane fraction by centrifugation of the material floated from homogenates of the slow growing 'WDA' tumour seem identical to those obtained by Prospero (1975). However, with the faster growing 'UA' tumour the pattern is quite distinct. The zone of plasma membrane sheets at the region of density 1.12-1.15 g/ml has fewer plasma membrane fragments, as indicated by enzymes such as 5'-nucleotidase, than the slow growing tumour. Both the yield and purification of plasma membrane fragments are higher for the slow growing tumour than for the fast growing tumour. This method is therefore more useful for isolating large sheet fragments of plasma membrane from the slow growing tumour. The reasons for
this observation were revealed by examination of the sections of 'UA' and 'WDA' tumours by electron microscopy, as will be discussed in the next section.

(b) Subfractionation of microsomes

As described earlier in Section III-2(c), I separated the plasma membrane vesicles, which are recovered in the microsomal fraction of hepatoma homogenates, by isopycnic flotation of microsomes in a linear sucrose gradient. The distributions through the gradients of a number of the commonly used plasma membrane marker enzymes were studied. With both 'UA' and 'WDA' hepatoma, plasma membrane fragments, as indicated by 5'-nucleotidase, alkaline phosphatase and alkaline phosphodiesterase, were separated from endoplasmic reticulum fragments, indicated by glucose-6-phosphatase and from lysosomes indicated by acid phosphatase. In contrast to the results with liver microsomes (Norris et al., 1974; Issa et al., 1977a), the distribution of 5'-nucleotidase was very similar to the distribution of other plasma membrane enzymes. With the 'WDA' tumour, all activities were found in a band centred at a density of 1.14 g/ml, which is very similar to the isopycnic banding density of the 5'-nucleotidase-rich 'sinusoidal' membranes in liver homogenate (Norris et al., 1974; Issa et al., 1977a). In the case of the 'UA' hepatoma, the plasma membrane fragments had a lower mean density and the very lowest density fragments appeared to be slightly enriched in 5'-nucleotidase and in alkaline β-glycerophosphatase.

Assay of marker enzymes does, however, show that the preparation of plasma membrane fragments of 'UA' hepatoma contained as contaminants other organelles such as endoplasmic reticulum and lysosomes. This observation is confirmed by the results obtained
after fractionation of membrane protein on polyacrylamide gel as described in Section III-2(d). The patterns showed a group of five bands which, by comparison with the distribution of enzymes, originate from the endoplasmic reticulum.

Tabulation of the purification and yield of various marker enzymes for both tumours (Tables III-5 and III-6) show that when using isopycnic flotation techniques for fractionation of the 'microsomal' fraction, the fast growing 'UA' tumour gave more purified plasma membrane fragments than the slow growing 'WDA' tumour. This explanation is consistent with the appearance of the plasma membrane in sections of the two hepatomas examined under the electron microscope (Section III-2(a)). Sections from the 'WDA' hepatoma showed a region of plasma membrane whose structure resembles that of the bile canaliculi in liver cells. No regions resembling bile canaliculi were found in the sections of the 'UA' hepatoma. The large sheets of plasma membrane present in liver homogenates derive largely from the bile canalicular face of the hepatocytes. So, the large sheets of plasma membrane recovered in the homogenates of 'WDA' hepatoma could derive from the region of the tumour cells which resembles bile canaliculi.

I have discussed the methods used for the purification of the large sheets of hepatoma plasma membrane and the small plasma membrane-derived vesicles which are recovered in the microsomal fraction. In discussing the results obtained with tumours, it is interesting to compare purification and specific activity with those obtained with normal liver. My experiments gave lower purification for hepatoma plasma membrane than the purification achieved by Issa et al. (1977b) with normal liver plasma membrane. This may be due to
tumour cells being smaller than hepatocytes. However, Dnistrian et al. (1975) who worked on the Morris 5123 tC hepatoma have also reported higher purifications than were obtained in this study. Thus it is likely that lower purification of tumour plasma membrane obtained in the present study may be due to the contamination of the plasma membrane preparation by other organelles such as lysosomes and endoplasmic reticulum fragments.

Extensive comparison of the enzyme activities displayed by plasma membrane isolated from rat liver and rat hepatomas was made by Emmelot and Bos (1969). They showed that the alkaline phosphatase activity of the hepatoma 484 (using p-nitrophenylphosphate as substrate) was higher than that on the liver, while the 5'-nucleotidase activity of the hepatoma was lower than that of the liver. In the present study both tumours show a higher activity for the alkaline p-nitrophenylphosphatase than was found in normal liver by other workers in this laboratory; this is consistent with those results reported by Emmelot and Bos (1969), although I did not myself measure alkaline phosphatase in the normal liver plasma membrane (Table III-2). Plasma membranes from both hepatomas show significant alkaline β-glycerophosphatase activity which is not detected in the liver membranes. Similar results were reported by Emmelot and Bos (1969).

The activity of 5'-nucleotidase in the 'WDA' hepatoma homogenate is similar or lower than that of a liver homogenate, while in the 'UA' hepatoma homogenate the activity of 5'-nucleotidase is higher than that of liver homogenate. Histochemical studies on early transplants of the 'U' hepatoma by El-Aaser (1965) showed that the belts of connective tissue which extend through the tumour are
rich in 5'-nucleotidase. Much less 5'-nucleotidase was detected in the tumour cells. Therefore, greater amounts of 5'-nucleotidase in the 'UA' hepatoma homogenate as compared to the liver and 'WDA' hepatoma homogenate do not necessarily mean that there is more 5'-nucleotidase in the plasma membrane of the 'UA' hepatoma cells.

Recently, it has been reported that two different membrane regions are obtained during preparation of liver plasma membrane (Wisher and Evans, 1975; Issa et al., 1977a; Caz and Evans, 1977). In the present study it was found that after homogenisation, the plasma membrane of hepatoma cells was recovered in two distinct forms, large sheets of membrane and vesicles. Wisher and Evans (1975) have reported that in the liver the membrane sheets derive largely from the bile canalicular face of the hepatocyte and the vesicles are derived from the sinusoidal surface. In this study it was found that the 'WDA' hepatoma gave sheets of plasma membrane and, on electron microscopy, was found to possess bile canalicular-like regions in the tumour. The 'UA' hepatoma gave very few sheets of plasma membrane and contained no bile canalicular-like regions. It is therefore reasonable to assume that the 'sheets' of plasma membrane recovered from the 'WDA' hepatoma derive, like the sheets of plasma membrane in liver homogenate, from the bile canalicular face of the cells. Because there are significant amounts of tumour plasma membrane in vesicular form, it becomes necessary to purify the microsomal fraction of the plasma membrane when dealing with the tumour. This is normally not the approach with liver, because sheets of plasma membrane give much higher purification.
IV-2 STUDIES ON MATERIALS DERIVING FROM THE PLASMA MEMBRANE

(a) Enzymes

The fractionation of proteins on Sephadex G-200 depends on the molecular weights and shape of the molecules. When the serum of 'UA' tumour-bearing rats was fractionated on Sephadex G-200 there was a clear peak for 5'-nucleotidase in the fractions corresponding to the 'void volume' of the column, suggesting that a small quantity of this enzyme is associated with large complexes with a molecular weight of more than one million. There was no trace of a similar peak in the serum of normal rats. Sepharose 4-B was used in an attempt to determine the molecular weight of the 5'-nucleotidase-carrying material which is excluded from Sephadex G-200. However, fractionation of the serum of normal and 'UA' tumour-bearing rats on Sepharose 4-B showed no significant differences, in particular no peak corresponding to the material excluded from Sephadex G-200 for 5'-nucleotidase. As the amount of 5'-nucleotidase activity in the complex eluted in the void volume of Sephadex G-200 is rather small, it would probably remain undetected if spread over several fractions on chromatography on Sepharose 4-B. Due to lack of time I was not able to repeat the experiment using large amounts of material.

The only conclusion that can be drawn is that the complex is not excluded from Sepharose 4-B and hence has a molecular weight of less than $4 \times 10^6$. If it were excluded, then one would expect a sharp peak of activity, as found with Sephadex G-200. The enzymes found in the complex detected by chromatography on Sephadex G-200 are known to be located in the plasma membrane. Shinkai and Akedo (1972) have reported a similar complex in animals and humans with hepatic cancer and presented evidence for the presence of a common
antigenic determinant in the complex and in the plasma membrane of the liver. This supports the suggestion that the complex could be a fragment of the plasma membrane released from the liver. These authors point out that liver tissue is rich in vascular beds which may increase the possibility that the plasma membrane of dying cells will be released into the blood circulation.

A non-immunological method for examining the surface of the 5'-nucleotidase-containing complex in the serum of tumour-bearing rats is suggested by studies on the properties of the enzymes described in Section III-4. Those results showed the effect of a variety of buffers of various pH on the 5'-nucleotidase and alkaline \(\gamma\)-nitrophenylphosphatase in the serum and in liver plasma membrane. The similarity of the changes of serum 5'-nucleotidase and alkaline \(\gamma\)-nitrophenylphosphatase indicates that the activities are due to a single enzyme. The enzymes found in the plasma membrane were, however, quite distinct in their properties. Hence, by careful choice of assay conditions, it should be possible to distinguish between the 'normal' serum 5'-nucleotidase and any additional activity deriving from the plasma membrane of liver or hepatoma cells.

(b) Immunological studies

Immunological studies on the serum of 'UA' and 'WDA' tumour-bearing rats after immunoelectrophoresis against anti-serum showed only slight differences as compared with the pattern obtained with normal serum, although other experiments (see Section IV-3) show that the serum of 'UA' tumour-bearing rats contains a specific protein which does not react with anti-(normal rat serum).
Immunoelectrophoresis against anti-liver plasma membrane does not show any qualitative differences in the proteins of normal rat serum and the serum of tumour-bearing rats and normal rats, but, as explained in Section III-1(b), there are quantitative differences in the amounts of proteins 'A' and 'B', the two serum components which react most strongly with anti-(liver plasma membrane). The area of peak 'A' is greater than peak 'B' in the serum of tumour-bearing rats, whereas in normal serum this situation is reversed. This is interesting in that protein 'A' would appear to be the $\alpha_2$SB globulin (Saba, 1978) which is involved in the non-specific removal of material from serum by macrophages.

The serum of 'UA' tumour-bearing rats also shows a distinct protein band when fractionated on polyacrylamide gel gradient (Section III-1(c)). This is the injury macroglobulin which is essentially absent in the normal serum (Fig. III-8). Ganrot (1973) has called this band the $\alpha_2$-acute phase globulin, and Gordon (1976) has isolated this $\alpha_2$-macroglobulin from $\alpha_1$-macroglobulin and shown that it is immunologically unrelated. Further, the serum of the 'WDA' tumour-bearing rat shows a clear band after fractionation on polyacrylamide gel gradient and staining for glycoproteins using periodic acid and Schiff's reagent (Fig. III-9(c)). The molecular weight of this protein suggests that it could be $\alpha$-foetoprotein which is reported to be present in some tumours and the serum of tumour-bearing animals (e.g. Alexander, 1972; Aoyagi et al., 1977). In any case, this band indicates the presence of a tumour-specific protein which might be released from the hepatoma plasma membrane. This point is discussed in more detail in the next section.
Changes in Proteins of Tumour-Bearing Rats

Immunoelectrophoresis of the serum of rats bearing the 'UA' and 'WDA' hepatomas against an antisera prepared against the 'UA' hepatoma plasma membrane, shows that there are three proteins in the serum of tumour-bearing rats which are either absent from normal rat serum or are markedly changed in their properties. The properties of the three proteins (marked as 'A', 'B' and 'C' in Fig. III-21) have been discussed in Section III-3(b). Protein 'A' appears as a double peak in all sera but the proportion of the rapidly moving component is markedly greater in the serum of tumour-bearing rats than in normal rat serum. Protein 'B' appears only in the serum of tumour-bearing rats. Protein 'C' is present only in the serum of 'UA' tumour-bearing rats and is absent from the serum of normal and 'WDA' tumour-bearing rats. It is probably identical to the protein in the serum of 'UA' tumour-bearing rats which reacts with anti-'UA' serum and not with anti-(normal rat serum). This observation confirms that, as discussed in the previous section, the serum of 'UA' tumour-bearing rats contains one protein which is absent from normal serum.

As shown in Fig. III-23(a)(b), neither protein 'B' nor 'C' react with the anti-(liver plasma membrane. Although protein 'B' does react with anti-(rat serum) the very strong reaction with anti-(tumour plasma membrane) suggests that it contains some tumour-specific antigenic determinants. This would suggest that protein 'B' is a complex formed by reaction of material released from the tumour with normal serum proteins.

Recently, there have been extensive studies on the immunological response of the host to a tumour. Investigators have worked both on the tumour-specific transplantation antigens (TSTA) and on
proteins such as α-fetoprotein, which are found both in foetal cells and tumours but not in adult animals (e.g. Thomson et al., 1973; Alexander, 1974; Eccles et al., 1976; Aoyagi et al., 1977; Akeson et al., 1977). The tumour specific transplantation antigens (TSTA) are defined as antigens that can produce specific rejection of tumour grafts by immunised animals (see review of Schmidt-Ullrich and Wallach, 1978). They are inferred to be plasma membrane components because the immune reactions known to produce tumour rejection must take place at the cell surface. The tumour specific antigens appear to be proteins never produced by normal animals, even in foetal life. For example, Schimdt-Ullrich et al. (1977) have reported the presence in the plasma membranes of GD 248 cells of at least three antigens not detectable in the membranes from the normal cell, which show no detectable cross reactivity with embryonic material.

Although it is clear that, at least in experimental animals, tumours do display specific antigens which can be recognised by this host, the tumours are not rejected. It has been reported (Thomson et al., 1973) that the antibody activity in the serum of tumour-bearing animals is masked by the release of soluble TSTA which forms antigen-antibody complexes with specific antibodies. The suggestion has been made that in the presence of excess antigen the migration of immunoblasts from lymph nodes may be blocked (Hall and Morris, 1965). However, immunoblasts are only one component in the cell-mediated response of the host against the tumour; small lymphocytes and macrophages are also involved. Thomson et al. (1973) have reported that it is possible that the circulating antigen and/or antigen-antibody complexes may, by directly interacting with the lymphoid cells or macrophages, interfere with their capacity to kill the specific
target tumour cell. Eccles et al. (1976) reported an observation that in rats with an HSBPA sarcoma, approximately $10^8$ monocytes/day must enter the tumour; normal rats have a turnover rate of around $3.6 \times 10^6$ monocytes/day and therefore to supply the monocytes entering the tumour their output has to be vastly increased. The stimulus may be provided by a factor released from the tumour which acts on the bone marrow, or be associated with the immune stimulus provided by the tumour-specific antigens. It is clear now that there is a factor releasing from the tumour-cell surface.

The results obtained in this study showed some evidence for tumour-specific antigens. Since protein 'A' is present in both normal rat serum and the serum of tumour-bearing rats, it is not relevant as a tumour-specific antigen. As discussed above, protein 'B', which appears in the serum of tumour-bearing rats but is not present in the serum of normal rats, although it does react with anti-(normal rat serum), may be a complex containing tumour-specific antigens. Protein 'C' which is present in the serum of 'UA' tumour-bearing rats has many of the properties of $\alpha$-fetoproteins. The presence of these proteins in serum is consistent with the proposition of Alexander (1974), that the rapid shedding of transplantation antigens is a characteristic of embryonic cells and tumours. Such shedding of antigens also occurs in human cancer, for example Aoyagi et al. (1977) have pointed out that $\alpha$-foetoprotein from foetal serum and from the ascites fluid of a patient with hepatoma had very similar structures.

Fractionation of the serum of tumour-bearing rats gave evidence for a 5'-nucleotidase-containing complex similar to that reported by Shinkai and Akedo (1972). Further examination of the pH curves suggests that the activities of the serum alkaline $p$-nitrophenyl-
phosphatase and 5'-nucleotidase are due to a single enzyme and excluded material from the Sephadex G-200 column did not show any alkaline \( p \)-nitrophenylphosphatase activity. Thus, it would seem likely that the high molecular weight of 5'-nucleotidase in the serum of tumour-bearing rats is not due to absorption of serum 5'-nucleotidase on to aggregated material, but derives from some other source, possibly the plasma membrane of tumour cells.
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