AN ANALYSIS OF GLYCOPROTEINS SYNTHESIZED BY HUMAN BREAST.

A thesis submitted for the degree of

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by

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"..what do you possess which you have not received and, if you receive it as a gift, why take the credit to yourself."
(1 Corinthians 4:7, The Holy Bible.)

DECLARATION

I declare that the work described herein is original and unless otherwise noted in the text or references has been conducted wholly by the author in the Department of Pathology, University of Leicester, during the period from October 1985 to August 1989.

PHILIP D. RYE.

PREFACE

This thesis describes the work done by the author in the department of Pathology from October 1985 to August 1989.

An introductory chapter guides the reader through glycoprotein structure and synthesis, describes the alterations that occur with malignancy, introduces the normal and pathological aspects of the mammary gland, before outlining the current state of biochemical markers in breast carcinoma. A brief section concludes this chapter by explaining why the work was done and listing the overall aims of the research.

The following five chapters, based on defined areas of work, begin with a brief introduction. This is followed by a number of sections in chronological order relating to the techniques used within the given area of work. (To avoid repetition between chapters, some of the techniques are cross referenced). These sections are sub-divided according to the specific aspects of each technique and, in addition to containing details of the materials and methods, there are comments where applicable on why particular methods were used. The remaining two sections of each chapter are devoted to the results and discussion pertaining to the particular area of study.

A final chapter collates the individual findings of chapters 2 to 6, concluding and placing in

context the significance of this study to breast cancer research.

"When we fence off limited fields of knowledge for special study, the missing context must be remembered, or our knowing is precocious and distorted and we end by knowing less, not more."

Derek Kidner (1985)

Acknowledgement.

I would like to express my gratitude to Professor Ian Lauder and all the members of the Pathology Department for their practical and vocal support throughout the period of my research studies here. In particular I am indebted to my supervisor Dr. Rosemary Walker who has demonstrated patience, encouragement and reproof with even temper and has expertly guided me to a greater understanding as a research scientist. To Dr. David Corcoran 'my-partner-in-crime' for the many constructive discussions and criticisms which have aided my progress thus far.

Finally I would like to thank Laura my wife who has put up with my extra marital affair with this thesis and patiently followed and supported me through my despair and discouragement and who now shares my relief that the end is in sight.

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ABSTRACT

Previous studies of glycoprotein structure and synthesis in breast cancer have used cell lines or animal models with limited analysis of primary tissues. Previous histochemical approaches have shown tumour associated alterations, although specific information relating to glycoprotein structure and size was not available.

This study analyzed the glycoproteins secreted and/or shed from non-malignant and malignant breast tissue and established human breast cancer cell lines. Major changes associated with malignancy were identified with glycoprotein size and fucosylation. A group of high molecular weight glycoproteins was analyzed further using immunological and biochemical techniques, and shown to be of similar protein core structure. The expression of a fucosylated glycoprotein of 230kD was of particular note since it was detected in 70% of carcinomas, six breast cancer cell lines, but no benign tissues. It was also one of a small number of glycoproteins that appeared susceptible to hormone modulation. Studies with the glycosylation inhibitor tunicamycin suggested that these glycoproteins contain

predominantly asparagine linked oligosaccharide chains.

A polyclonal antiserum (P5252) was raised to one of the glycoproteins of this group and used to screen a number of normal and malignant human tissues. Competition studies and immunoblotting showed that the antiserum recognized antigens not detected with other antibodies directed against the milk fat globule membrane, breast tumour antigens and fibronectin. Attempts to purify the glycoprotein from primary tumours was unsuccessful which neccessitated the use of breast cancer cell lines.

Analysis of plasma membranes, to determine the probable location of the glycoproteins, highlighted the difficulties of working with primary tumour tissue having a high stromal content. Preliminary analysis of membrane preparations from cell lines was also done.

This work has shown many differences in the glycoproteins released from benign and malignant human breast, in particular a tumour specific fucosylated glycoprotein of 230kD which is worthy of further study.

CHAPTER 1

INTRODUCTION

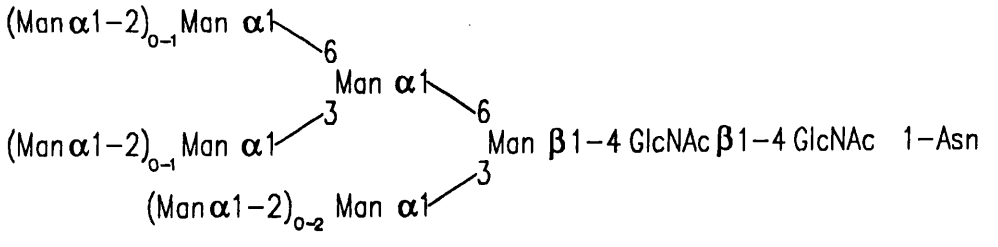
1.1. Glycoproteins.

1.1.1.Glycoprotein structure.

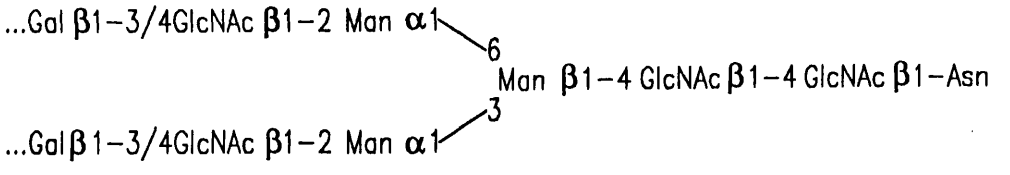
Glycoproteins are proteins that contain oligosaccharide chains covalently attached to specific amino acid residues. They range in molecular weight from 15000 daltons to over one million, and the carbohydrate structures typically range from disaccharides to very complex structures incorporating 18 or more monosaccharide units (oligosaccharides). An excellent text on the subject of glycoprotein structure can be found in

Figure 1.1. Examples of the three main types of N–linked oligosaccharide chains. Simple or high mannose (a), complex (b) and hybrid type (c).

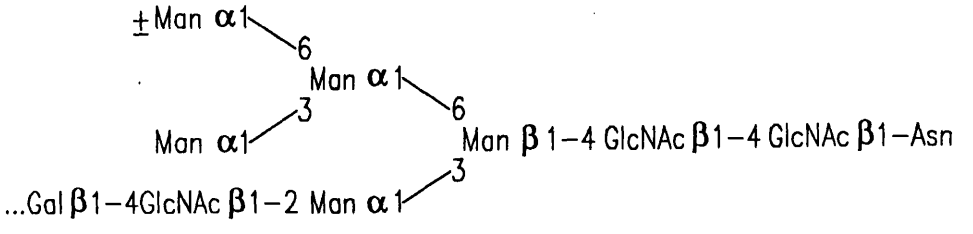
(a).



(b).



(c)



"Glycoproteins" by R.C. Hughes (1983). The monosaccharides commonly occurring in glycoproteins are the neutral sugars mannose (Man), galactose (Gal) and fucose (Fuc), the amino-sugars (hexosamines) glucosamine and galactosamine and their acetylated derivatives, N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc), and the sialic acids (Sial) of which N-acetylneuraminic acid (NeuNAc) is an example.

The most common type of sugar-peptide linkage involves the amino group of an asparagine (Asn) residue in the sequence Asn-X-Ser/Thr (where X is any amino acid), and the C1 of a saccharide, N-acetylglucosamine. The N-acetylglucosamine then becomes the site of attachment of other monosaccharide residues. This type of linkage is referred to as the N-glycosidic linkage.

These N-linked sugar chains, or glycans, consist of a common core structure with the sequence:

Manβ1→4GlcNAcβ1→4GlcNAc→Asn

Structurally they can be subdivided into three groups; simple or high mannose type, complex type and hybrid type. The simple type contain only mannose and N-acetylglucosamine residues. The core structure of seven sugar residues is characteristic of this subgroup and variation is achieved by the number and location of Manα1→2 residues (shown in parenthesis in figure 1.1a.) linked to the three non-reducing terminal αmannosyl residues. These high

mannose type structures tend to be more common in lower organisms. The complex type glycoproteins, (figure 1.1b.) found only in the higher animals, contain a greater variety of residues. Structural variation thought to be species specific is achieved by the number, location and type of residue attached to the non-reduced αmannosyls of the core structure. Although many outer chains often contain the trisaccharide sequence:

NeuAcα2→6Galβ1→4GlcNac

the major source of structural diversity comes with the attachment of the terminal sugars, sialic acids, fucose, galactose, N-acetylgalactosamine and N-acetylglucosamine.

The third subgroup, established after an investigation into the larger sugar chains isolated from hen egg albumin (Yamashita, *et al.*, 1978), is the hybrid type, (figure 1.1c.). This group as the name suggests comprise structural features of both the high mannose and the complex type sugar chains.

The second and less frequent type of sugar-peptide linkage is the O-glycosidic linkage. This involves a glycosidic bond with the free hydroxyl side chains of serine (Ser) or threonine (Thr). It is thought that a specific tripeptide sequence may not be necessary, although the absence of any secondary structure may allow better accessibility of the transferase to the reaction site (Hill, *et al.*, 1977). The most common sugar

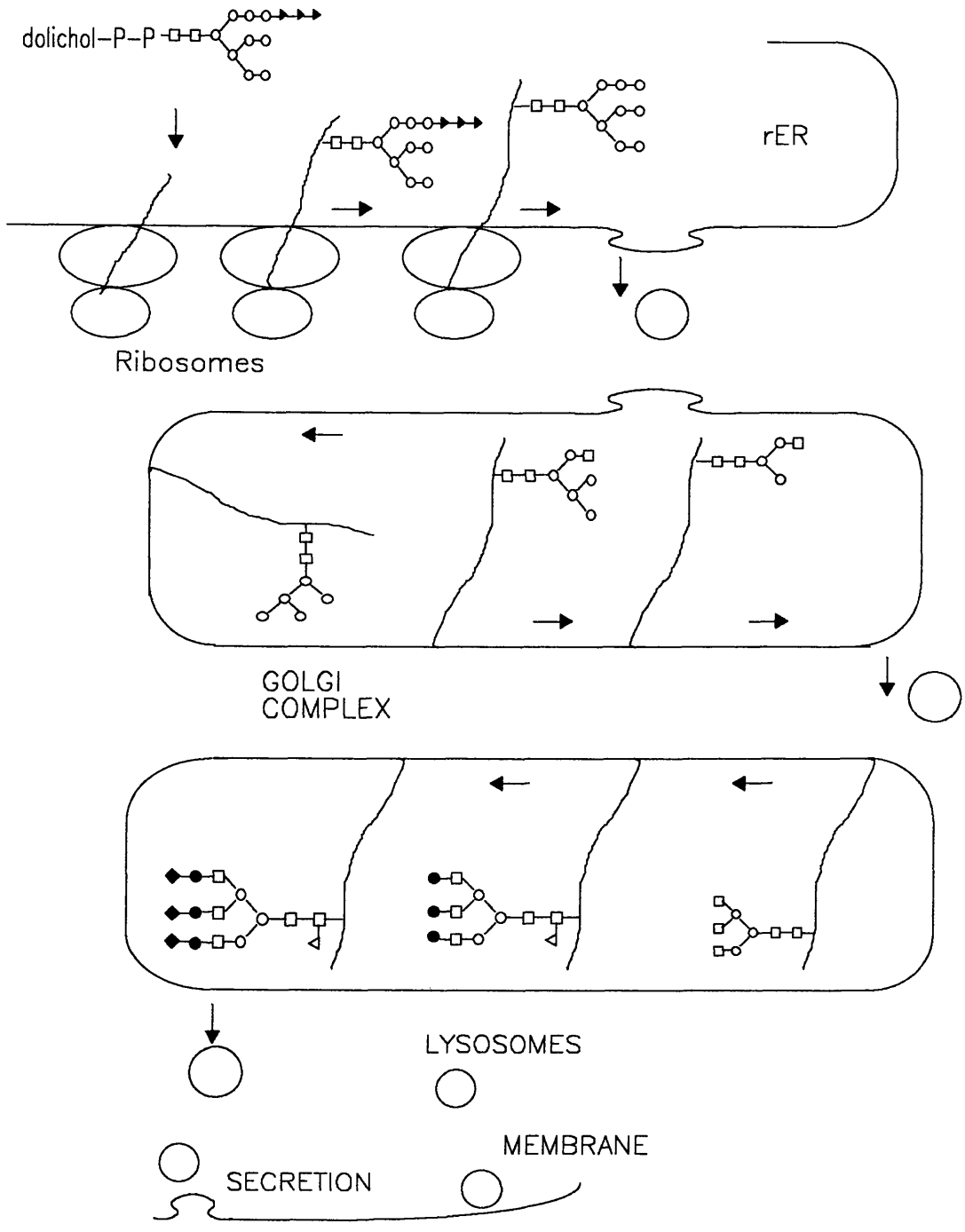
residue found in this linkage is N-acetylgalactosamine. These O-linked glycoproteins are commonly known as mucins, due to their abundance in this proteinaceous material, and play vital roles as extracellular lubricants e.g., saliva, digestive and urinary tract secretions. These viscous mucinous secretions also serve as protective barriers against mechanical or chemical insult. Other O-linked glycoproteins include the blood group substances and components associated with the milk fat globule membrane (see section 1.3.1.).

1.1.2. Glycoprotein synthesis.

From 1975 to 1980 major advances were made in our understanding of the biosynthetic pathways of glycoprotein assembly. This complex multistep process involves many highly specific enzymes located throughout the rough endoplasmic reticulum (ER) and Golgi complex. Glycosylation of the N- and O-linked glycoproteins is slightly different and will therefore be described separately.

N-linked glycosylation. This begins by the synthesis of a lipid linked oligosaccharide precursor dolichol phosphate (P-Dol). The biosynthesis of this precursor is carried out by membrane bound glycosyltransferases in the rough ER and has been called the Dolichol Phosphate Cycle (Sharon & Lis, 1982). The resulting structure is transferred *en bloc* to the exposed asparagine residues of the

Figure 1.2. Probable sequence for N–linked oligosaccharide chain processing: □ = N–acetylglucosamine
o = mannose residues
► = glucose residues
▷ = fucose residues
◆ = sialic acid residues



polypeptide chain being synthesized in the rough ER (figure 1.2.). Transfer only occurs at asparagine residues in the tripeptide sequence Asn-Y-Ser/(Thr) (Struck & Lennarz, 1980) although asparagine in this sequence is not always glycosylated.

Once translation of the polypeptide is complete, soluble glycoproteins and glycoproteins destined for secretion are extruded completely into the ER cisternae. Glycoproteins predetermined to be membrane located remain integrated in the ER membrane. This latter state is achieved by the presence of a hydrophobic membrane spanning sequence within the polypeptide. These partially glycosylated glycoproteins are transferred to the Golgi (Palade, 1975; Rothman & Fine, 1980) where further glycosylation changes are made possible by the many specific hydrolases and transferases present in this organelle. Soon after the transfer of the high mannose core to the polypeptide, hydrolysis occurs and 3 glucose residues are removed (Hubbard & Ivatt, 1981). This results in a glycoprotein with a high mannose type oligosaccharide structure. Synthesis of the complex type oligosaccharides involves the removal of all but 5 of the mannose residues. Continued processing at this stage first requires the addition of N-acetylglucosamine (Tabas & Kornfield, 1978; Harpaz & Schachter, 1980) which, if no further processing occurs, will result in a hybrid type oligosaccharide structure described earlier. Further enzymatic cleavage of the remaining

mannose structure, leaving the $Man_3GlcNAc_2$ core, leads the way forward for the sequential addition of N-acetylglucosamine, fucose, galactose and sialic acid residues. The structure resulting from this process would be a complex type oligosaccharide.

O-linked glycosylation. Unlike the process outlined for N-linked glycosylation there appears to be no intermediate reaction between polypeptide synthesis and individual sugar transfer (Hanover, *et al.*, 1980). Glycosylation begins with the transfer of N-acetylgalactosamine to the hydroxyl group of threonine or serine of the newly synthesized polypeptide. Subsequent glycosylation appears to continue by the sequential addition of saccharide residues in the presence of specific transferases.

The site of this initial pathway in the glycosylation of the O-linked glycoproteins is still uncertain. Whereas with N-linked glycoproteins, glycosylation can be initiated on the newly synthesized polypeptide in the rough ER, this is not likely to be the case for the majority of O-linked glycoproteins: several studies have shown the levels of the N-acetylgalactosamine transferase to be a factor of 10 lower in the rough ER than in smooth ER or Golgi (Kim, *et al.*, 1971; Ko & Raghupathy, 1972; Hanover & Lennarz, 1980).

Glycosyltransferases. The enzymes responsible for the biosynthetic assembly just described are known

collectively as the glycosyltransferases and are responsible for the stepwise synthesis of both N- and O-linked glycoproteins. These enzymes catalyse the synthesis of specific glycosides by the transfer of a monosaccharide residue from a donor substrate to an acceptor substrate. The donor substrates are usually nucleotide sugar derivatives although the lipid linked sugars dolichol-phosphoryl-mannose (Dol-P-Man) and dolichol-phosphoryl-glucose (Dol-P-Glc) are also used. The acceptor substrate may be another monosaccharide residue, a polypeptide or a lipid, depending on the type of transferase involved. The donor substrate however is usually a diphosphonucleoside.

The glycosyltransferases may be grouped according to the type of sugar transferred, thus all galactosyltransferases transfer galactose from uridine diphosphate galactose (UDPgal) to their specific acceptors, while neuraminyltransferases transfer neuraminic acid from its cytidine monophosphate donor (CMPNeu) to the required acceptor. Further specificity is derived from the enzymes ability to recognize certain acceptor sequences and not others. This can be demonstrated with an example:

βGalactosidase α2→6sialyltransferase incorporates sialic acid exclusively into α2→6 linkage with galactose in the oligosaccharides containing the non-reducing terminal sequence, Galβ1→4GlcNAc. However transfer does not occur if

the non-reducing terminal sequence is Galβ1→3GlcNAc-αThr/Ser or GalNAc-αThr/Ser. The remarkable specificity expressed by these enzymes gave rise to the "one linkage-one enzyme" hypothesis (Hagopian & Eylar, 1968; Schachter & Roden, 1973; Schachter & Roseman, 1980).

1.1.3. Membrane glycoproteins.

In 1976 it was suggested that all membrane proteins are glycosylated, i.e. they are *glyco*proteins (Gahmberg, 1976). Since no evidence to the contrary has been forthcoming this view is generally accepted. Glycoproteins associated with the cell membrane may be divided into two categories, *peripheral* or *extrinsic* proteins and *integral* or *intrinsic* proteins. This discrimination is based on the ease with which the proteins are dissociated from the cell membrane. Peripheral proteins require only mild treatments, such as an increase in the ionic strength or the presence of a chelating agent. These proteins dissociate in a molecularly intact form, free from lipids, and they remain soluble in neutral aqueous buffers. As their name suggests peripheral proteins are only weakly associated with the surface of the membrane. Conversely integral proteins have a very much stronger association, which is reflected in the harsh procedures necessary to remove them from the membrane. Integral proteins that span the bilayer have a continuous hydrophobic stretch of 20-28 amino

acids which lack the charged residues arginine, lysine, glutamic acid, aspartic acid, histidine and proline. These membrane spanning proteins maintain their structural and functional asymmetry because these hydrophobic domains cannot be dislodged from the stable environment of the lipid bilayer. Integral proteins can only be removed after treatment with detergents, e.g. Triton X, bile acids, protein denaturants or organic solvents. Furthermore they remain associated with lipids despite their isolation from the membrane, and remain insoluble or aggregated in neutral aqueous buffers.

There are many characteristics of cell surface located carbohydrate that make them ideal for a variety of membrane mediated functions. They are hydrophilic and exclusively located at the outer face of the membrane and are therefore accessible. The enormous variety of possible structures within the carbohydrate chains enables them to have a wide range of groups available for highly specific interactions: for example 3 molecules of the same hexose can form 176 different trisaccharides, while 3 molecules of the same amino acid can only form 1 tripeptide. Indeed, small structural changes can and do translate to large functional differences in cell behaviour. This is demonstrated clearly in blood groups where the recognition of A and B types of the ABO blood group system, differ in carbohydrate structure only in a single N-acetylated amino group.

Since Gesner and Ginsburg's (1964) work on membrane function in lymphocytes, it has been recognized that there is a strong association between membrane glycoproteins and many cell-surface mediated functions which include **cell recognition and adhesion, membrane transport** and **membrane receptors.**

Cell recognition and adhesion. Recognition of foreign tissue in a host is determined by the major histocompatability complex (MHC) (Snell, 1981). This group of genes encode for specific membrane glycoproteins which are thought to serve as antigen binding receptors. These glycoprotein receptors, once activated by a foreign antigen, help to initiate a specific lymphocyte response. Quite apart from the process of graft rejection, cell recognition and adhesion is of fundamental importance to the normal formation and function of tissues and organs. Fibronectin, a complex type glycoprotein that represents a majority of the cell surface glycoprotein in fibroblasts, is thought to have a specific function in cell-cell and cell-substratum adhesion (Vaheri, *et al.*, 1978; Hughes & Pena, 1978). Although it is widely accepted that glycoproteins are involved in a wide variety of cell-cell interactions it is still unclear as to their precise role. One theory is that based on Roseman's work (1970) where cells have both glycosyltransferases and their oligosaccharide

substrates on the plasma membrane. Adhesion would occur when complimentary oligosaccharide acceptors and transferases on individual cells came together to form an enzyme-substrate complex. The specificity of the transferases (section 1.1.2.), make them attractive candidates for these surface located acceptors. It was further suggested that with the transfer of a sugar residue to the oligosaccharide acceptor molecule, the enzyme-substrate complex would dissociate and the cells separate resulting in an altered cell surface. This mechanism of a controlled dissociation of the enzyme-substrate complex is attractive since it may explain phenomena such as contact inhibition of growth. Experimental data from a variety of cell systems has showed support for this cell surface transferase theory; neural retina cells (Roth, *et al.*, 1971), blood platelets (Bosmann, 1971; Barber & Jamieson, 1971a; 1971b; Bosmann, 1972), algal gametes (McLean & Bosmann, 1975) and cultured fibroblasts (Lloyd & Cook, 1974; Spataro, *et al.*, 1975;). However this theory is not universally accepted.

The most popular alternative to the membrane transferase theory are membrane located sugar receptors, or lectins (Goldstein, *et al.*, 1978). These membrane lectins, highly specific for carbohydrate structures, are thought to be involved in many interactions between cells and soluble or membrane bound complementary carbohydrate structures (Simpson, *et al.*, 1978; Monsigny, *et al.*, 1983).

Recent studies using monoclonal antibodies to these lectins could prove to be a useful tool in evaluating their role, not only in normal cellular interactions, but also in tumour cell behaviour and metastasis (Raz, *et al.*, 1984).

Membrane transport. The structure of the apolar membrane lipid bilayer facilitates its role as a protective barrier. The hydrophobic property of this bilayer inhibits large, and especially water soluble molecules, from passing through, the regulation of which is vital in the maintenance of cytoplasm composition. However the membrane must not be absolute in this function as a barrier if it is to fulfil the requirements of a living cell, and thus it must be maintained as a selectively permeable barrier. This selective permeability is achieved by the presence of specific glycoproteins in the bilayer. Transport of some small molecules and ions across the lipid bilayer is achieved by specialized transmembrane glycoproteins, each being specific for individual or closely related molecules.

Membrane receptors. An important membrane function is the receipt and dispatch of various intercellular signals such as hormones. Some of these hormones are by nature lipid soluble and are therefore free to cross the membrane in order to bind to their nuclear receptors. Most other hormones or transmitters are either small hydrophilic (non-lipid soluble)

molecules such as adrenaline and acetylcholine, or polypeptides such as epidermal growth factor, insulin and glucagon, which all bind to specific receptors on the cell membrane (Cuatrecasas, 1974; Todaro, *et al.*, 1976; Fabricant, *et al.*, 1977; Das, *et al.*, 1977). The transmitter substances are generally thought not to pass directly through the membrane, but to trigger a secondary effect inside the cell via membrane receptor protein (Cuatrecasas, 1974; Michaelson and Raftery, 1974; Czech, 1977). The pituitary hormone TSH (thyroid stimulating hormone) binds several glycolipids at its receptor site and it is thought that this constitutes part of the TSH receptor complex (Mullin B.R., *et al.*, 1976).

As mentioned earlier the ABO blood group isoagglutinins are highly specific for their glycoprotein counterparts on the erythrocyte. Another blood group antigen located on a surface glycoprotein is the MN system. Studies of this sialylated glycoprotein, glycophorin A, have shown that M and N antigenic specificities probably involve both carbohydrate and amino-terminal amino acids (Lisowska & Duk, 1975)

The classical ABO(H) blood group system has been well characterized (Watkins, 1980). The antigenic determinants are found not only on erythrocytes but also on many other cells and as a soluble form in a variety of cell secretions. The soluble antigenic forms are glycoproteins, the blood

group specificity of which is determined by the combinations of individual sugar residues at the oligosaccharide chain ends.

The site of viral attachment in the erythrocyte membrane has been extensively studied. Viral attachment is effected by the presence of glycophorin (a major sialoglycoprotein) in the plasma membrane, and terminal sialic acid residues as well as other parts of the glycoprotein have been shown to be involved in virus binding. It is not clear what happens on other mammalian cell membranes but it is likely that they similarly involve a membrane glycoprotein.

1.1.4. Secreted glycoproteins.

Unlike the apolar hydrophobic regions which anchor the membrane proteins to the lipid bilayer, secreted proteins in their final form exhibit a mainly polar surface. Proteins destined for secretion are synthesized in the rough ER and subsequently glycosylated during their passage through to the trans Golgi stack (lysozomal proteins excepted).

Newly synthesized glycoproteins destined either for location at the cell surface or for secretion, leave the trans Golgi stack via transport vesicles to the plasma membrane. The vesicles fuse with the membrane releasing the soluble glycoproteins into the extracellular environment and exposing the membrane glycoproteins to the outer

surface. This transport can occur by two mechanisms, constitutive or regulated secretion (Tartakoff & Vassalli, 1978). The constitutive mechanism is said to be involved if secretion is equal to the rate of synthesis, i.e. there is no storage of synthesized material prior to secretion. Conversely the regulated mechanism involves the storage of material destined for secretion, at high concentration, until the cell receives an appropriate stimulus allowing it to fuse with the plasma membrane.

The structural and functional polarity of many cells make it important that secretory vesicles containing membrane or secretory products, are targeted to the correct membrane surface. A suggestion of specific receptors and "signals" interacting with cytoskeletal components has been proposed (Blobel, 1980), although the mechanisms surrounding this event are still far from clear.

1.2. Alterations Occurring with Malignancy.

1.2.1. General pathology.

Cell proliferation in normal tissue is strictly controlled, the mass of any tissue being maintained at a constant level. Cell division and proliferation will only occur in order to replace lost or damaged cells within that tissue. Under certain normal conditions a tissue may increase in size, due to cell proliferation or cell hypertrophy,

as a result of increased physiological demand upon that tissue. However once demand has ceased the tissue reverts to its original size. The process is reversible. Neoplasia however is an irreversible pathological process of excessive cell proliferation, the extent of which is not beneficial to the host. The result of this malregulated growth gives rise to a mass of tissue called a tumour or neoplasm. In the tumour, proliferation increases beyond these normal limits such that the mass of the tissue increases irreversibly. The rate at which this proliferation occurs varies from tumour to tumour and in some cases it is very slow, but it always exceeds the rate of normal cell replacement.

Tumours are divided into one of two major categories, benign and malignant. The benign tumour remains as a localized single mass within the tissue from which it originated and tends to grow very slowly over a period of years. This type of tumour rarely causes problems unless it exerts pressure on adjacent structures or is involved in the excessive production of hormones such as islet cell pancreatic tumours that secrete insulin. These types of tumour are usually outside the normal regulatory feedback mechanisms. Nevertheless benign tumours are usually successfully treated by surgical removal. The malignant tumours, more commonly termed cancers, tend to be less well differentiated than the benign tumours. The prime distinction however is that the malignant cell is not restricted to its immediate

surroundings. The cells of a malignant tumour can invade adjacent tissue structures. Furthermore they can invade and penetrate the endothelial lining of the blood and lymphatic circulation. This may result in further tumour growths elsewhere in the body. These secondary tumours are termed metastases and the manner in which they dissociate from the original tumour site and invade and grow at distant sites is called the metastatic process. In order to produce metastases tumour cells must be capable of overcoming several restrictive physiological barriers. These include: (i) dissemination from the primary tumour; (ii) survival of local defences; (iii) local invasion of surrounding tissue; (iv) penetration of lymphatic and/or blood vessel linings; (v) survival of the mechanical and physiological stresses in the circulation; (vi) evasion of leucocyte defence mechanisms; (vii) arrest and "binding" to the capillary endothelium of a "target" organ; (viii) penetration of capillary endothelium; (ix) invasion of local tissues; and (x) proliferation at secondary site. This complex cascade of steps is thought to be performed by very few of the total number of tumour cells within the primary lesion (Fidler, 1978). Indeed it has been noted that of those cells capable of local invasion, few possess the ability to penetrate the circulation. Similarly of those cells able to reach the circulation fewer still are able eventually to re-establish tumour growth at a distant site

(Fidler, 1978; Poste & Fidler, 1980; Fidler & Nicolson, 1981). The metastatic cell, against all odds, becomes the "*decathlon winner*".

1.2.2. Molecular aspects.

Most of the studies that have identified molecular alterations in malignancy have been done with cultured cells *in vitro*, often resulting in cells that are said to be transformed. The term transformation however, is defined strictly as a cellular alteration *in vitro*, that would result in cell growth in a manner similar to that of a genuine tumour derived cell, again in vitro.

Although cell growth in this manner may result in cell changes that are not identical to those of the corresponding tumour cell *in vivo*, this method of investigation has nevertheless been useful. Moreover growing cells artificially allows the experimental parameters to be manipulated and controlled precisely. Subsequent cellular alterations can then be observed easily and associated with specific events in malignancy. Conversely observing malignant change *in vivo* cannot be controlled to the same extent because of the multiplicity of factors involved, such as the nutritional state of the whole animal, hormone balance, etc. It would therefore be more difficult to associate cellular alterations with specific events. The assumption therefore is that molecular alterations *in vitro* resulting in transformation,

are identical to those of the malignant cell *in vivo*. The term transformation, applied to the cell cultured *in vitro*, therefore may be regarded as analogous to the term tumourigenicity, applied to the malignant cell *in vivo*.

There are many alterations at the molecular level that lead to or result from malignant transformation: alteration in enzyme patterns; alteration in growth characteristics; cytological changes; growth factor production; synthesis of oncodevelopmental gene products; changes in cell membrane structure and function.

Alteration in enzyme patterns. As one may expect in the malignant cell, there are altered enzyme activities in the synthetic and degradative pathways of nucleic acid metabolism. In general the activities of the enzymes involved in DNA and RNA synthesis are increased and the corresponding catabolic enzymes are decreased (Weber, 1977a; 1977b). This trend is also seen in the enzymes of protein and amino acid metabolism: enzymes responsible for protein synthesis are increased; amino acid catabolizing enzymes are decreased. Elevated expression of specific proteases in some malignant tumours and cell lines is well documented (Ruddon, 1987). Indeed the malignant cells ability to migrate and invade its surroundings could be attributable to the action of proteases on specific cell-surface molecules involved, for example, in

adhesion.

The many and varied enzymes involved in the synthesis and processing of carbohydrates on glycoproteins are also altered by malignant transformation. Altered cellular glycosidase levels have been found in transformed mouse, chicken and hamster cells (Kijimoto & Hakomori, 1971; Bosmann, *et al.*, 1974). Furthermore, in human breast and colonic cancers there are higher levels of β-galactosidase, α-mannosidase and neuraminidase, than in adjacent normal tissues (Bosmann & Hall, 1974).

Alteration in growth characteristics. Probably the most useful attribute of the transformed cell is its "immortality", enabling cells in culture to live for an indefinite number of population doublings, while their normal counterparts, under the same conditions, have a limited life expectancy. Transformed cells with this "immortality" frequently undergo chromosomal or karyotypic changes characterized by an alteration in the number of chromosomes (aneuploidy/polyploidy). This alteration of chromosomal material may result either from *de novo* synthesis or as a result of selection during continued growth in culture. These changes may reflect the *in vivo* process since poorly differentiated malignant cells in humans often show this karyotypic change. Further to this altered growth characteristic, transformed cells also

exhibit a decrease in density-dependent inhibition of growth (Stoker and Rubin, 1967). This is most readily seen in culture where transformed cells "pile up" rather than stop growing when contact with other cells are made. There are two aspects to this phenomenom: firstly inhibition that results in a loss of cell movement; secondly, inhibition that results in a loss of cell replication. The presence of only one of these inhibitory control mechanisms will therefore not necessarily prevent the "pile up" phenomenom. In some non-transformed cells it has been shown that inhibition of movement occurs, yet these same cells grow to the cell density of their transformed counterparts, indicating inhibition of replication has been lost.

It is not certain that these inhibitory mechanisms are cell-contact related. Non-transformed cells may be metabolically prevented from further growth in dense cell cultures by a lack of available nutrients, oxygen or local pH changes. Transformed cells would be able to escape these localized effects due to their anchorage independant growth. Studies using virus transformed cells have shown that those cells forming tumours in mice, consistently showed this loss of anchorage dependant growth, whereas other cellular alterations had reverted to their non-transformed states (Shin *et al.*, 1975). This ability of transformed cells to grow without attachment to a surface is probably the one *in vitro* characteristic that is most closely

associated with tumourigenicity.

Cytological changes. Most of the cytological changes seen in the malignant phenotype of transformed cells are also seen in animal and human tumours *in vivo*. Some of these changes therefore are used as histological criteria to aid diagnosis of human tumours. The general cytological changes seen in malignant cells include, an increase in number and size of nuclei, abnormal mitosis eg. tripolar, loss of adhesion resulting in isolated cells, and formation of clusters or cords of cells.

Growth factor production. Growth factors are present in many types of tissue, both adult and embryonic, and are probably released by all cells in culture (Shields, 1978). They are substances (usually polypeptides) that specifically stimulate cell proliferation and often cell differentiation. Growth factors act in a paracrine fashion, diffusing locally through intercellular spaces and although many have been characterized according to their action on specific cell types, there is a growing body of evidence pointing to a more general range of activity (Sporn & Roberts, 1988).

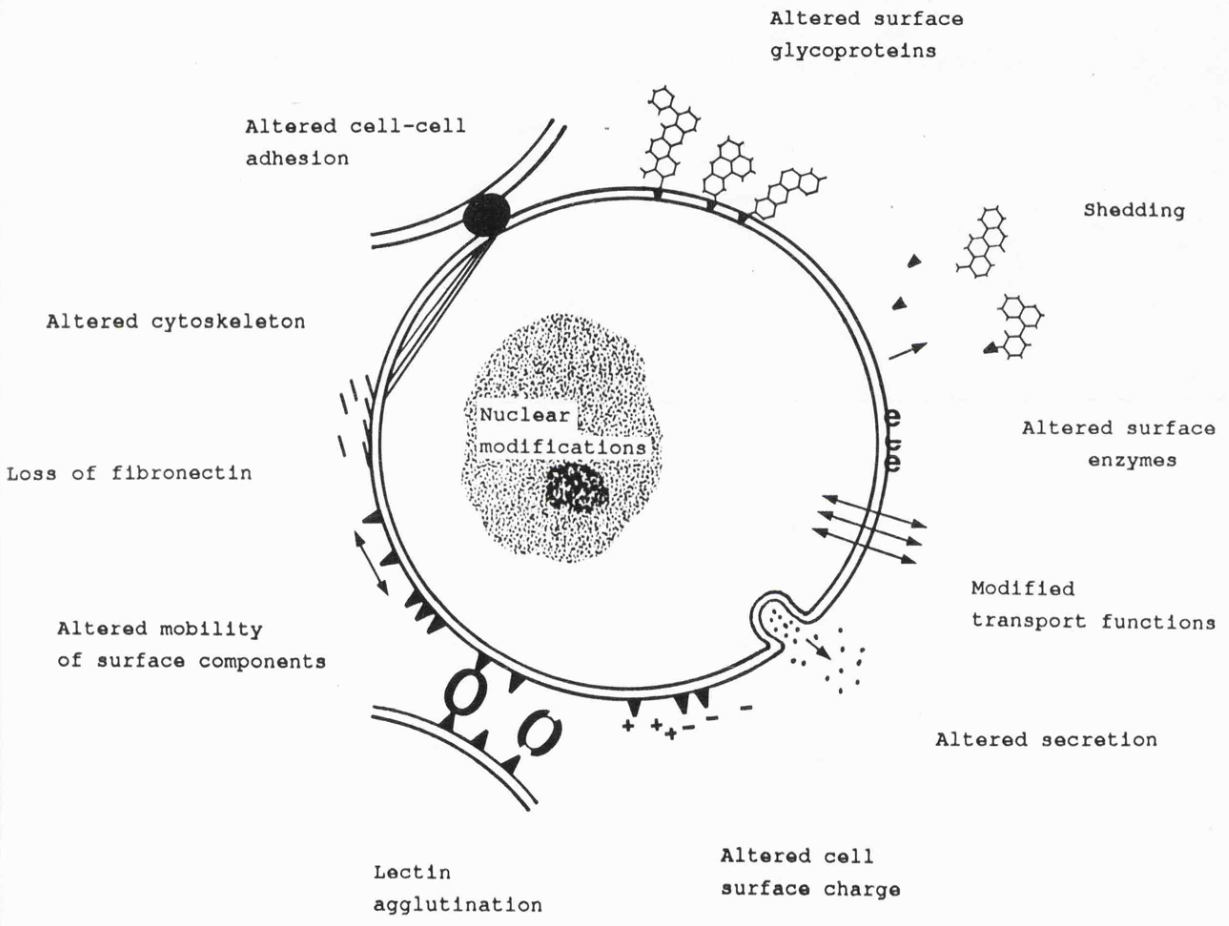
Evidence supporting the association of growth factors with malignancy was first proposed when transformed cells were shown to have a minimal requirement for them (Barnes & Sato, 1980; Kaplan, *et al.*, 1982). This altered requirement could be due

to the transformed cell (a) producing its own growth factor and therefore relying on an autocrine mechanism, (b) synthesizing a modified receptor or (c) by-passing the cellular pathways involved in growth factor signal transmission. Evidence for all three of these possible mechanisms has come from studies of oncogenes and oncogene products. Platelet derived growth factor (PDGF), which is the product of the c-sis proto-oncogene, is produced by many transformed mesenchymal cells (which are normally target cells for PDGF). Moreover certain transformed cells both produce and respond to transforming growth factor β (TGFβ) thereby indicating some sort of autocrine process. (Sporn, *et al.*, 1987; Centrella, *et al.*, 1987).

Recent studies indicate that the ras proto-oncogene protein is an essential intermediary in the intercellular transmission of the growth factor signal (Mulcahy *et al.*, 1985). The products of the myc and fos oncogenes are thought to interact in a similar manner. Therefore activation of the oncogenes ras, myc and fos in the transformed cell could produce the same or similar effect as that expected from growth factor stimulation. Indeed it has been shown in breast tissue that the frequency and elevated expression of oncogenes is increased with malignancy (Whittaker, *et al.*, 1986).

Changes in cell membrane structure. Many alterations in the plasma membrane that occur in malignancy can

Figure 1.3. Some biochemical alterations in the malignant cell.

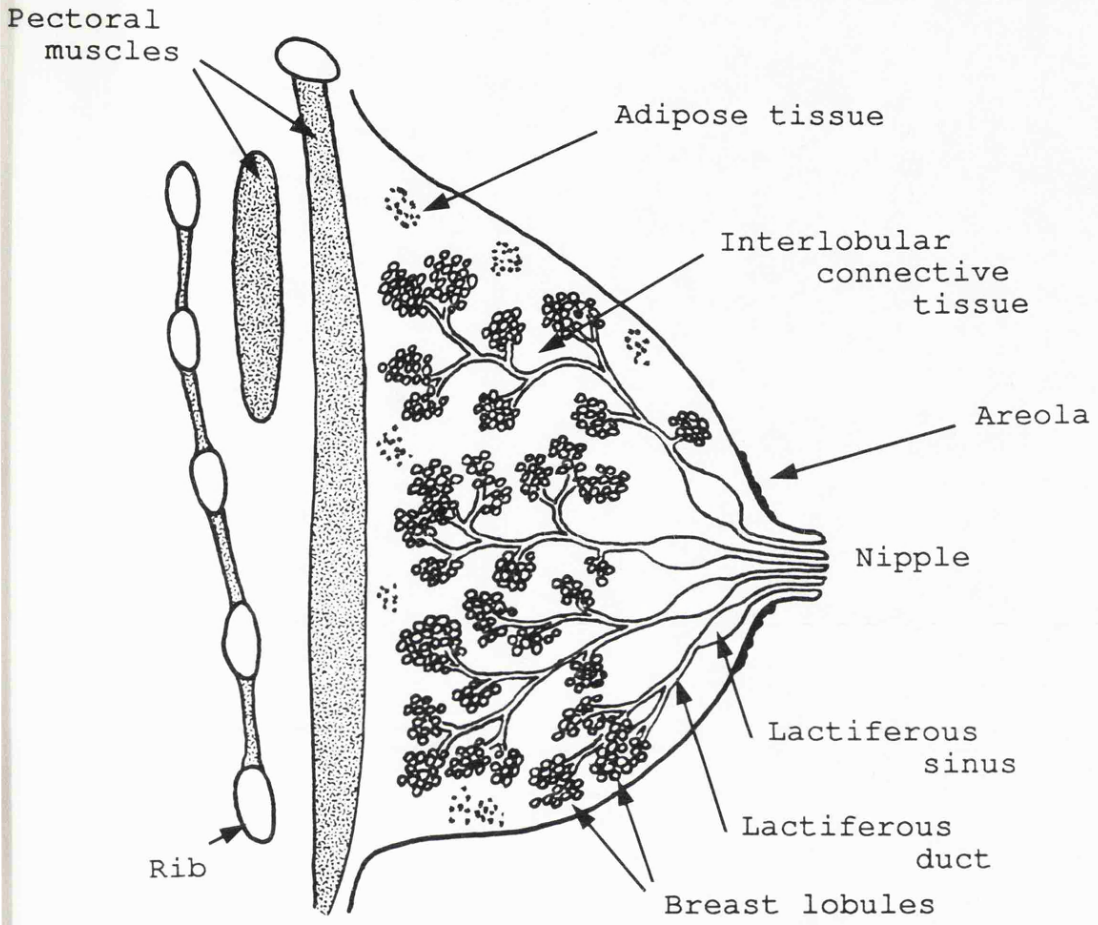


be inferred from some of the cellular characteristics previously mentioned e.g., loss of anchorage dependance and modified adhesion. Indeed many biochemical alterations of the malignant cells membrane have been observed and some of these are illustrated in figure 1.3.

Changes in the processing of carbohydrate side chains of cell surface glycoproteins during malignancy is well established (Feizi, 1985; Hakomori, 1985). Initially it was thought that these changes were solely due to an increase or decrease in sialic acid content which was reflected in the increased levels of sialyl transferases in transformed cells (Bosmann, *et al.*, 1968; Warren, *et al.*, 1972), although later studies indicated that these differences were more complex (Muramatsu, *et al.*, 1973; Ceccarini, *et al.*, 1975; Ogata, *et al.*, 1976).

Many studies have shown altered glycosylation with respect to the incorporation of specific saccharides. It has been observed that an increase in fucose-containing glycoproteins correlates with tumourigenicity (Glick, *et al.*, 1973; 1974). In addition there appears to be an increase in the molecular weight of those glycoproteins containing fucose (Santer, *et al.*, 1984). Changes in the processing of glycoproteins containing galactose and mannose have also been observed. Later studies using mouse teratocarcinoma cell lines (Ivatt, 1985) identified two distinct pools of high mannose

Figure 1.4. Structure of adult human breast.



glycoproteins, the distinguishing feature being the rate at which they are processed. This altered rate of glycosylation of membrane proteins has also been suggested by Yamashita *et al.*, (1985).

Besides the general alterations seen in glycoprotein processing with malignancy more specific changes have not only revealed the loss of existing glycoproteins (Yamada & Olden, 1978), but also the appearance of new high molecular weight glycoproteins (Bramwell & Harris, 1978). Although there have been a number of studies in this field of research, much of the work has used either animal model systems or human cell lines and there still remains a need to study human primary tumour tissues.

1.3. Breast And Its Pathology.

1.3.1. Normal breast structure and function.

The normal breast (figure 1.4.), begins to develop in the sixth week of foetal life. The mammary ducts grow down from the skin surface in a manner similar to the sweat glands. The female breast further develops after menarche under the influence of oestrogen (17β oestradiol) from developing follicles in the ovaries. The functional or secretory units of the breast are called acini which are located within the breast lobules or terminal duct lobular units (TDLU) and surrounded by

fibrous connective tissue. The small ducts connecting with the acini converge and the resultant ductules lead towards the nipple area where they join to produce the major lactiferous ducts. These in turn lead into the lactiferous sinuses before narrowing again to pass through the nipple. The entire ductule system including the acini are lined by a layer of epithelial cells. Underlying this is a layer of myoepithelial cells which is discontinuous in places. The myoepithelial cells in normal breast do not come into direct contact with the lumen of the ducts. The remainder of the breast is adipose (fatty) tissue. Blood supply to the breast comes from the thoracic branches of the axillary artery and from the internal thoracic and intercostal arteries. Venous drainage of the breast is via the axillary and internal thoracic veins. Lymphatic drainage of the breast occurs predominantly via the axillary lymph nodes which are situated close to the branch of the axillary mammary artery. Most of the remaining lymph drains via the parasternal nodes.

Such is the physiological structure and function of the human breast that its responsiveness to hormonal influence is not suprising. There are many changes in the breast that have been related to the fluctuating levels of hormones in the menstrual cycle. These changes have been defined in terms of cell ultrastructure (Fanger & Ree, 1974), cell turnover (Ferguson & Anderson, 1981) and cell secretory activity (Going *et al.*, 1988).

During pregnancy the breast, in preparation for lactation, undergoes significant alterations. The controlled proliferation and differentiation of the glandular structures, with an increase in size and number of acini and subsequent enlargement of the TDLU's, is paralleled by an increase in the synthetic and secretory activity of the glandular epithelium (Ferguson & Anderson, 1983). The latter changes are seen as an increased amount of rough ER and dilation of the Golgi apparatus.

The milk released from the breast during lactation contains a variety of substances in addition to the carbohydrate and lipid components. These include casein, the major protein component, αlactalbumin, one of two proteins responsible for lactose synthesis, and milk fat globule membranes. The latter component is derived from the luminal surface of the actively secreting epithelial cells. It has been the subject of many studies in breast tumour pathology and because of the relative ease in obtaining and preparing samples it has received much attention, not least as a potential prognostic marker in breast disease (section 1.4.2.), (Edwards, *et al.*, 1980).

At the end of lactation the breast returns to its normal resting state, a process called involution. In many cases however return to the resting state is incomplete and well developed TDLU's may persist for several years (Bonser, *et al.*, 1961). The involutionary changes also occur

with increasing age, with eventual replacment by fibrous tissue. This regression and subsequent loss of functioning glandular tissue was thought to occur primarily at the menopause although a recent study has shown regression from the third to the sixth decade (Hutson, *et al.*, 1985), indicating a gradual transition. Nevertheless many women still retain a large proportion of glandular tissue well into old age.

1.3.2. Benign breast disease.

Benign breast disease may occur in women of all ages and can be divided under two headings;

Benign Tumours	Other Benign Changes
Fibroadenoma	Cystic disease
Intraductal papilloma	Sclerosing adenosis
	Mammary duct ectasia
	Intraductal papilloma
	Epithelial hyperplasia (lobular)
	Epithelial hyperplasia (ductal)

The most common type of benign breast tumour in women is the fibroadenoma. It occurs predominantly in younger women, with the greatest incidence in the third decade. Fibroadenomas range in size usually from 1 to 4cm in diameter although

much larger tumours are found. They are well circumscribed and have no connections with the normal surrounding tissue. These tumours, as the name suggests (*-adenoma*), arise from the lobules and as a result of this may undergo certain hormonally induced changes e.g., during pregnancy when fibroadenomas tend to grow more rapidly. The majority of studies indicate that there is no increased risk of malignancy associated with fibroadenomas.

Benign intraductal papillomas can occur in women of all ages. They are usually solitary lesions and are frequently located in the large ducts near the nipple. A typical intraductal papilloma comprises a cauliflower-type structure arising from the duct wall with a central stalk of loose connective tissue and blood vessels.

There has been much controversy surrounding the increased risk of breast carcinoma in patients with benign breast disease. However it is now generally accepted that this increased risk or predisposition for malignancy is only associated with epithelial proliferative lesions (Dupont & Page, 1985). This benign disease involves proliferation of the epithelial cells lining the ducts and acini. It can occur in women of all ages although the incidence peaks in the 40-50 age group.

1.3.3. Carcinoma of the breast.

Carcinoma of the breast is the most frequent

cause of death from malignancy in European and American women, and its incidence in most Western countries is increasing (Haagensen, 1986). There is a geographical variation, with the lowest rates in S.E. Asia. It is the most common cause of death in women aged 35-40yrs, and in the United Kingdom alone over 15,000 women die each year. The incidence of the disease markedly increases with age, from 20/100000 at 30yrs, to 75/100000 at 40yrs and 175/100000 at 55yrs.

There have been a variety of factors associated with an increased risk of breast cancer; early menarche, late menopause, late pregnancy, nulliparity and hereditary links. However, the most striking of these is the latter, where it has been shown that there is up to a ninefold increased risk in first degree relatives of pre-menopausal women with cancer in both breasts (Anderson, 1974).

It is of special note that while the factors that are thought to elevate the risk of breast carcinoma are numerous there appears to be only one protective factor demonstrated so far which is an early full term pregnancy (MacMahon, *et al.*, 1973). Indeed this has been known since 1842 when Rigoni-Stern observed that breast carcinoma was more frequent in unmarried women and nuns! (Haagensen, 1986). The aetiology of breast carcinoma is far from clear and many predisposing factors have been implicated. The interaction of some of these factors and their possible association with risk groups are

Figure 1.5. Risk factors and mechanisms associated with malignancy.

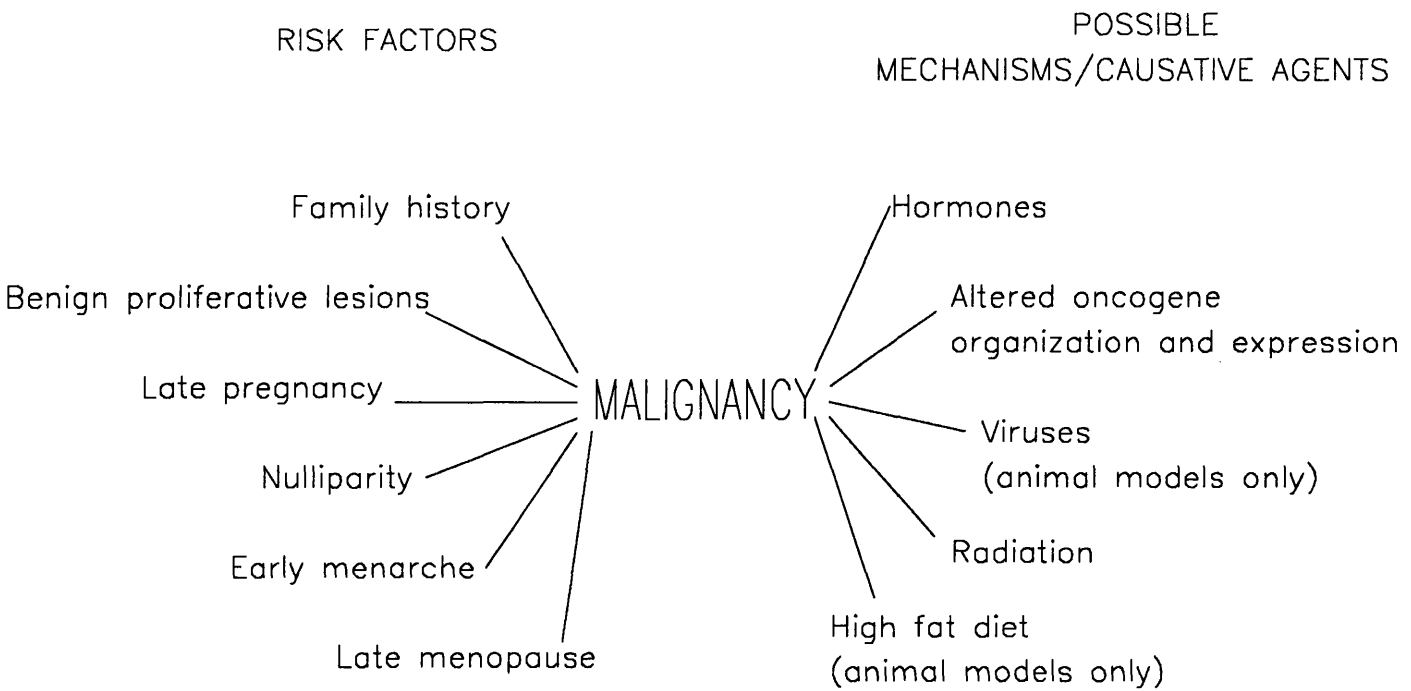
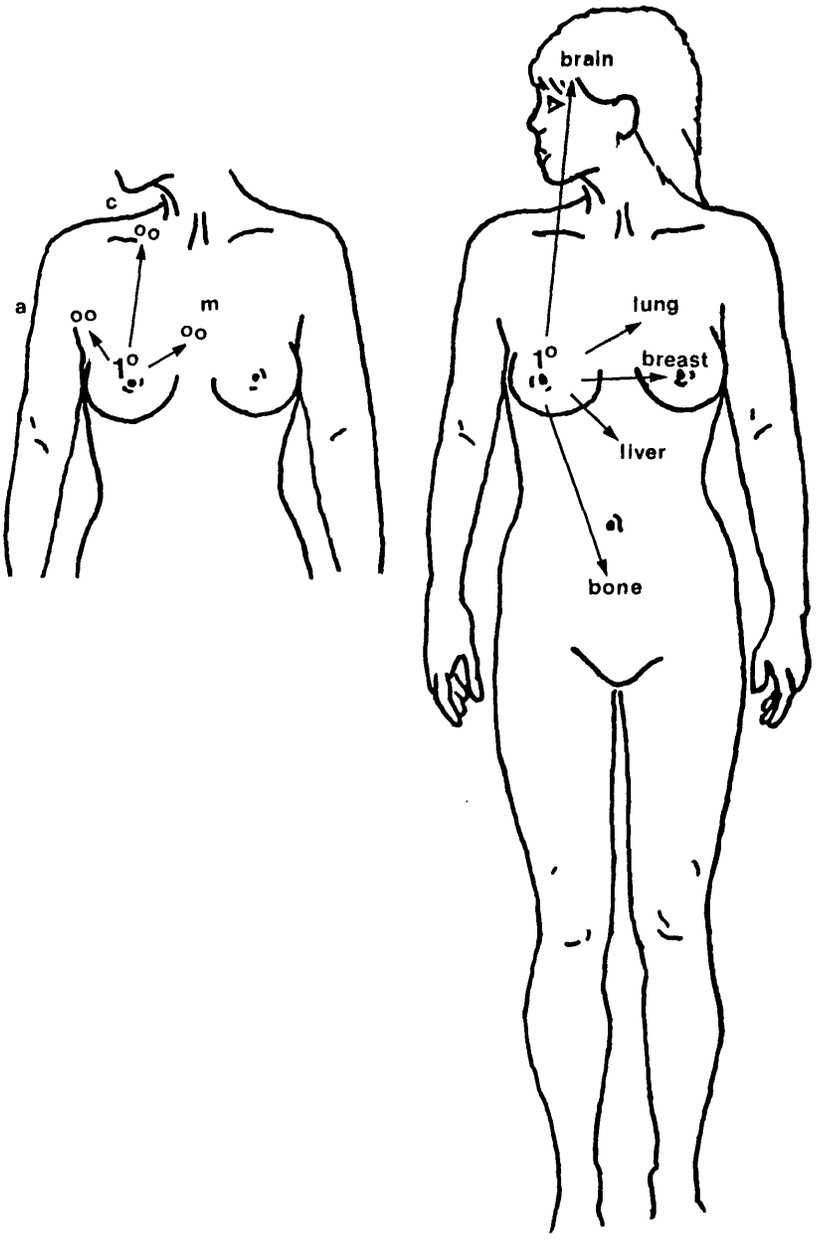


Figure 1.6. Metastatic spread of primary breast carcinoma. Lymph node involvement also shown; a= axillary lymph nodes, c= clavical lymph nodes, m= mammary or parasternal lymph nodes.



shown in figure 1.5.

Carcinoma of the breast is classified, according to WHO criteria, under two major headings, non-invasive and invasive (see footnote).[1] The tumour cells of a non-invasive carcinoma are confined either to the ducts or acini of the lobules and do not penetrate the basement membrane. The opposite is true for invasive carcinomas which as the name suggests, involves penetration of the basement membrane and spread of the tumour cells throughout the surrounding tissue. Metastatic spread of the carcinoma results in secondary tumours forming at distant sites (figure 1.6.). The various types of carcinomas are listed below.

Non-invasive Carcinoma	*Invasive Carcinoma*
Intraduct.	Invasive ductal.
Intralobular.	Invasive lobular.
	Mucinous.
	Medullary.
	Papillary.
	Tubular.
	Secretory.

The most common type of breast malignancy is invasive ductal carcinoma constituting 70% to 80% of

[1]A third category exists in the WHO report for Pagets disease but this is not covered within the range of this text.

Table 1.1. UICC AJC clinical staging system. (American Joint Committee on Cancer, 1987).

T		Primary Tumour.
T0		No evidence of primary tumour.
Tis		In situ cancer.
T1		Tumour diameter less than 2cm.
	T1a	No attachment to underlying pectoral muscle.
	T1b	Attachment to underlying pectoral muscle.
T2		Tumour diameter between 2-5cm.
T3		Tumour diameter greater than 5cm.
	T3a	No attachment to underlying pectoral muscle.
	T3b	Attachment to underlying pectoral muscle.
T4		Tumour (any size) in extending to chest wall or skin.
	T4a	Attachment to chest wall.
	T4b	Oedema, skin ulceration or satellite skin nodules.
	T4c	Both of the above.

N	Regional Lymph Nodes.
N0	No tumour in axillary lymph nodes.
N1	Tumour growth in axillary lymph nodes.
N2	As above and nodes fixed together or to other structures.
N3	Tumour growth in supraclavicular or infraclavicular nodes or oedema of the arm.

M	Distant Metastasis
M0	No evidence of metastasis
M1	Distant metastasis present and skin involvement beyond breast area.

Table 1.2. Comparison of UICC-AJC and Manchester staging system.

Manchester Clinical Stage Grouping	UICC-AJC Clinical Stage Grouping		
Stage I	T1	N0	M0
Stage II	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
	T2	N1	M0
Stage IIIa	T3	N0	M0
	T3	N1	M0
	T0-3	N2	M0
Stage IIIb	T4	N0-3	M0
	T0-4	N3	M0
Stage IV	T0-4	N0-3	M1

all mammary carcinomas (Rosen, 1979), and can occur in both pre- and post-menopausal women. Histologically the tumour cells are arranged in cords and gland-like structures and the extent of cell differentiation can vary between individual tumours of the same type. The well differentiated infiltrating ductal carcinoma, showing cells in a gland-like pattern, will behave less aggressively than a poorly differentiated tumour where the cells appear as solid sheets. The amount of connective tissue or stroma between the tumour cells can also differ, resulting in a high degree of histological variation.

1.3.4. Tumour stage and grade.

For a clinician to plan effective treatment for a patient with breast cancer a thorough knowledge of the extent of the disease is required; this is known as staging. There are currently two staging systems in use, the UICC-AJC or TNM system and the Manchester staging system. These systems employ alpha-numeric nomenclature and are summarized in tables 1.1 and 1.2.

Assessment of differentiation (grading) can provide useful information about the likely behaviour of tumours. The grading system that was employed throughout this work was the Bloom and Richardson method with the modification as described by Elston *et al.*, (1982). This grading divides tumours into three categories: well differentiated

(grade I); moderate (grade II); and poorly differentiated (grade III). The method considers three aspects; the degree of tubule formation, the extent of nuclear pleomorphism and the mitotic count.

1.4. Biochemical Markers of Breast Carcinoma.

1.4.1. The need for tumour markers.

> "...few authors who have set out to seek such differences have returned empty handed. The real problem is not to find differences, but to determine which of the many differences that have been found, are responsible for the malignancy, that is, for conferring on cells the ability to grow progressively and kill their host." (Bramwell & Harris, 1978).

Although Bramwell and Harris recognized the legion of altered characteristics involved in malignancy they appear to negate the value of these differences as biological markers from a clinical perspective. Many tumours have the ability to produce a wide variety of molecules, some of which may determine a degree of specificity for abnormal cells. However, to date few have been discovered.

The ability to demonstrate these markers in tumour tissue or body fluids in the early stages of disease would be a great asset in patient management, most notably in the prediction of

behaviour, estimation of tumour load, localization and detection of metastases, and response to therapy. In the past morphological criteria have dominated the histopathological classification of tumours. However whilst some tumours may show similar morphology the disparate expression of these molecules may indicate differences in biochemical cell behaviour, metastatic potential, prognosis and response to therapy.

When a tumour is diagnosed it is important to establish the cell type or tissue of origin of the tumour (Gatter, *et al.*, 1985). This is particularly important in discriminating between the primary tumour and its metastases. The use of antibodies against normal tissue markers has helped some diagnoses, particularly in distinguishing tumours of non-epithelial origin, lymphomas and sarcomas, from carcinomas (epithelial tumours). Moreover, an essential prerequisite for successful therapy is that the tumour is diagnosed correctly (Ramaekers, *et al.*, 1983). Monoclonal antibodies against tumour markers have a number of potential uses. If a tumour produces a specific component then antibodies raised against this can be labelled with a radio-opaque tag and subsequently used to localize, not only the primary but also secondary growths of tumour at a very early stage. A more direct therapeutic aid has been to label these anti-tumour-marker antibodies with cytotoxic molecules, e.g., the cytotoxic lectins ricin and abrin (powerful inhibitors of

cellular protein synthesis), which could then be used to selectively kill only those cells expressing the specific tumour marker. Work by Houston and Nowinski (1981) using a ricin-linked murine monoclonal antibody directed against the Thy1.1 antigen, showed a high degree of specificity. However this method of therapy, somtimes referred to as the "*Magic Bullet*", is still in its infancy. Nevertheless it does have the advantage of avoiding the aggressive chemical and radiological techniques currently in use. A major stumbling block to this line of therapy is the lack of specific tumour markers.

Over the last ten years the search for tumour markers has received a great deal of interest. Although no single biochemical marker exists for breast carcinoma, studies have shown a variety of substances elevated in the body fluids, predominantly serum, of these patients (Franchimont, *et al.*, 1976; Coombes, *et al.*, 1977; Coombes, 1978; Cove, *et al.*, 1979). Further approaches have involved the use of whole cell homogenates (Horne, *et al.*, 1976), cell culture lines (Schultz & Ebner, 1977) and immunohistochemical techniques (Walker, 1979; 1982).

Biochemical markers associated with breast carcinoma can be separated into two groups, (a) substances that are appropriate to breast, i.e., found in normal breast tissue, these may act as markers of differentiation, and (b) substances which

are inappropriate to breast i.e., expressed only in the tumour.

1.4.2. Appropriate or differentiation markers.

The milk protein αlactalbumin is synthesized only in differentiated breast epithelial cells. It is involved in the synthesis of lactose from glucose in the production of milk (Fitzgerald, *et al.*, 1970). The levels of this protein appear to be hormonally regulated with increased amounts during lactation and a decrease at involution. Although the presence of this protein is indicative of functional differentiation there is no consistent relationship with histological differentiation, lymph node metastasis and hence prognosis. Indeed a comparison of the studies involved with the immunohistological detection of lactalbumin in breast carcinomas (Walker, 1984) varies considerably, 0% (Bailey, *et al.*, 1982), 37% (Bahu, *et al.*, 1980), 51% (Walker, 1979), 61% (Clayton, *et al.*, 1982) and 70% (Bussolati, *et al.*, 1977). A further two milk proteins have also been mooted as potential differentiation markers, casein (Monaco, *et al.*, 1977; Cowen, *et al.*, 1978) and lactoferrin (Hurlimann, *et al.*, 1976) although it is now generally accepted that these milk proteins are of little value in the prognosis of breast carcinoma (Walker, 1984; Cohen, *et al.*, 1987).

Some of the discrepancies seen in the results from these different research groups may be

Table 1.3. Monoclonal antibodies against breast tumours.

Antibody	Antigen	Immunogen	Reference
Mc5	high Mr gp.	HMFGM	Ceriani, et al., (1983)
HMFG-1	300-400 kD gp.	HMFGM	Arklie, et al., (1981) and Burchell, et al., (1983)
HMFG-2	80-400 kD gp.	HMFGM	Taylor-Papadimitriou, et al., (1981)
MAM-6	>400 kD gp.	HMFGM	Hilkens, et al., (1984a; 1984b)
MAM-3	surface high Mr gp.	HMFGM	Hilkens, et al., (1984a), and Gooi, et al., (1985a; 1985b)
M8	high Mr gp.	HMFGM	Foster, et al., (1982a; 1982b) Foster and Neville, (1984) and Gooi, et al., (1985a; 1985b)
M18	surface gp.	HMFGM	(as above)
M24	39-59 kD gp.	HMFGM	Gooi, et al., (1985a; 1985b)
E29	265-400 kD gp.	HMFGM	Cordell, et al., (1985) and Heyderman, et al., (1985)
B72.3	220-400kD gp.	Tumour cells	Colcher, et al., (1981) and Nuti, et al., (1982)
NCRC-11	high Mr gp.	Tumour cells	Ellis, et al., (1984) and Price, et al., (1985)
DF3	290 kD gp.	Tumour cells	Kufe, et al., (1984)
3E1.2	>300 kD gp.	Tumour cells	Stacker, et al., (1985; 1988)
MBE6	cytoplasmic	Lymph node metastasis	Schlom, et al., (1980) and Teramoto, et al., (1982)
CF 29/34	cytoplasmic	Lymph node metastasis	Imam, et al., (1985)
312C-1	Cytokeratin	Secondary breast culture	Dairkee, et al., (1985)
MBr1	high Mr gl.	Cell lines, MCF-7	Menard, et al., (1983), Caneari, et al., (1983) and Mariani-Constantini, et al., (1984)
H59	surface gp.	ZR-75-1	Yuan, et al., (1982) and Hendler, et al., (1985)
F 36/22	high Mr mucin	MCF-7	Papsidero, et al., (1983; 1984) and Croghan, et al., (1983)
24-17.2	100 kD ?gp.	MCF-7	Thompson, et al., (1983)
AB/3	?glycolipid	MCF-7	Iacobelli, et al., (1985)
SP-2	90kD proteolipid	MCF-7	Iacobelli, et al., (1986)
3B18	cytoplasmic	MCF-7	White, et al., (1985)
15A8	surface	MCF-7	(as above)
45-B/B3	cytokeratin	MCF-7	Karsten, et al., (1983)

attributed to their individual polyclonal antisera. Raising monoclonal antibodies using the technology of Kohler and Milstein (1975) avoids this problem. This approach has been pursued by several groups (Table 1.3), with respect to the milk fat globule membrane (MFGM) and several monoclonal antibodies have been raised using this as an immunogen in order to identify possible prognostic markers (Edwards, *et al.*, 1980; Arklie, *et al.*, 1981; Taylor-Papadimitriou, *et al.*, 1981; Foster, *et al.*, 1982a; 1982b; Hilkens, *et al.*, 1984a; Foster, *et al.*, 1982a). These antibodies have been further evaluated immunohistochemically with regard to prognosis (Wilkinson, *et al.*, 1984; Berry, *et al.*, 1985; Rasmussen, *et al.*, 1985). Two of the antibodies that have received a considerable amount of attention are HMFG1 and HMFG2 (Taylor-Papadimitriou, *et al.*, 1981). Both these antibodies recognise different epitopes on a group of high molecular glycoproteins present in normal human milk (Burchell, *et al.*, 1983). HMFG1 reacts predominantly with lactating breast and epithelial cells cultured from milk. HMFG2 however reacts with a determinant expressed more strongly in carcinomas of the ovary and breast (Burchell, *et al.*, 1983; 1985) than normal epithelial cells which may reflect the altered glycosylation pattern of these tumours. Indeed the value of the HMFG2 antibody has been demonstrated in the radio-imaging of ovarian carcinomas in humans (Granowska, *et al.*, 1984;

Pateisky, *et al.*, 1985), and also in the treatment of metastatic carcinoma (Hammersmith Oncology Unit, 1984). However more detailed analysis of the epitopes to which these antibodies bind is required for them to be of independant prognostic value (Turnbull, *et al.*, 1986).

Other approaches to identifying differentiation markers have involved the hormone receptor proteins present in breast carcinoma cells. A large proportion of this work has concentrated on the oestrogen receptor and oestrogen regulated proteins (Greene, *et al.*, 1980; McCarty, *et al.*, 1980; Westley & Rochefort, 1980). A proposed biochemical mechanism of the relationship between the hormone and its effect has been described (Osborne, *et al.*, 1980). The behaviour of breast tumour cells is thought to be related to oestrogen activity, but only about 50% of those patients expressing the oestrogen receptor actually respond to hormone therapy (McGuire, *et al.*, 1975). One of the markers of oestrogen action in breast carcinoma is the progesterone receptor which is itself regulated by oestrogen (Namer, *et al.*, 1980). It is used in conjunction with the oestrogen receptor for determining a course of therapy.

Because of the need to identify patients who will respond to therapy of this kind, an oestrogen regulated protein could prove to be a potentially useful marker. The presence of a 52000 dalton glycoprotein secreted from human breast carcinoma

cells (Westley & Rochefort, 1980) appears to be secreted only by malignant cells. This glycoprotein has been shown to be stimulated by oestrogen (Veith, *et al.*, 1983) although further studies are necessary to confirm its usefulness as a marker in breast carcinoma.

1.4.3. Inappropriate or tumour markers.

The absence of differentiation seen in tumour cells following malignant transformation is probably the reason why some tumours express products of an oncofoetal origin (substances primarily found in foetal tissues and fluids). The carcino-embryonic antigen (CEA), a glycoprotein with a molecular weight of 200000 daltons, is one such product. The incidence of this glycoprotein in breast carcinoma has been shown to range widely, from 2% to 83% (Goldenberg, *et al.*, 1978; Heyderman & Neville, 1977; Shousha, *et al.*, 1979; Walker, 1980). This varied incidence of CEA is reflected in its complex heterogeneity (Rogers, 1976; Alpert, 1978). CEA shares antigenic determinants with closely related substances (Von Kleist, *et al.*, 1972; Von Kleist, 1979) and as a result, polyclonal antisera show a significant degree of cross-reaction. Although its value as a marker in breast carcinoma is limited (Chu & Nemoto, 1973; Cohen, *et al.*, 1987), it does appear to be useful as a marker in colorectal carinoma (Mackay, *et al.*, 1974; Neville & Cooper, 1976).

Other possible tumour markers that have been studied in breast include the placental glycoproteins, human chorionic gonadotrophin (HCG) (Braunstein, *et al.*, 1973) and β-1 pregnancy specific glycoprotein (Horne, *et al.*, 1976). However the widely ranging incidence of both these glycoproteins has resulted in them being of limited prognostic value.

The use of hybridoma technology with breast carcinoma cell lines and membrane enriched preparations of metastatic tumours has given rise to a number of monoclonal antibodies (Table 1.3). One of these is B72.3, which has been shown to react with about 50% of breast carcinomas (Nuti, *et al.*, 1982) and over 80% of colon carcinomas (Stramignoni, *et al.*, 1983) with no reactivity observed in a variety of normal tissues. However the most promising monoclonal antibody raised so far is NCRC11 (Ellis, *et al.*, 1984) which recognises a glycoprotein with a molecular weight greater than 400000 daltons (Price, *et al.*, 1985). This antibody reacts in a similar pattern to antibodies previously raised to components of the HMFGM. Recent studies show there is a strong correlation between the extent of NCRC11 reactivity in breast tumour tissue and an extremely good prognosis (Ellis, *et al.*, 1984; 1987).

Despite a variety of approaches many of the antibodies that have been raised detect a similar group of high molecular weight glycoproteins

(Griffiths, *et al.*, 1987). Although these are expressed to a much greater extent in carcinomas, they are also present in normal and benign breast epithelium. It would appear that there is further scope for the identification of more and better human tumour markers.

1.5. Research Justification and Aims.

There is a large volume of evidence implicating glycosylation modifications of cell surface glycoproteins associated with maligant transformation of cells (Warren & Buck, 1980; Smets & Van Beek, 1984; Hakomori, 1985). These alterations could be of significance in determining tumour cell behaviour since cell surface glycoconjugates have been implicated in many cell-cell and cell-substrate interactions (Olden, *et al.*, 1982). Many of the studies which have examined glycoprotein structure and synthesis in human tumours have been confined to malignant cell lines (Lloyd, *et al.*, 1974; Schwartz *et al.*, 1986; and Debray, *et al.*, 1986), rather than primary neoplasms, and comparisons with normal cells has been limited.

The identification of glycosylation changes in human breast carcinomas could be of importance with regard to the recognition of tumour associated markers and in the prediction of tumour behaviour. Previous histochemical studies using lectins confirmed that there are differences in specific

sugar groups between the glycoproteins of benign and malignant human breast and that some alterations are related to tumour differentiation or to some extent metastatic potential (Walker, 1984a; 1984b; 1984c; 1985). Heterogeneity in the in vitro utilization of radioactive labelled sugars by malignant human breast in comparison to normal has also been found by using tissue autoradiographic techniques (Walker, *et al.*, 1986). However these histological approaches cannot give information about differences in glycoprotein structure and size occurring with malignancy.

The aim of this work therefore was to: 1) identify specific glycosylation differences between benign and malignant breast lesions, 2) characterize the differences in terms of glycoprotein structure composition and size, and 3) determine their relevance or significance in the context of tumour cell behaviour.

The techniques used to attain these aims are described in the following five chapters. At the begining of each of these chapters is a short introduction giving the reasons why a particular approach was used and outlining some of the main problems associated with that approach.

CHAPTER 2

COMPARATIVE ANALYSIS OF GLYCOPROTEINS RELEASED FROM BENIGN AND MALIGNANT HUMAN BREAST IN-VITRO.

As detailed in section 1.5 the identification of glycosylation changes in human breast carcinomas could be of importance with regard to the recognition of tumour associated markers and in the prediction of tumour cell behaviour. In order to identify some of these glycosylation changes in human primary breast, the glycoproteins were metabolically labelled (for an extensive review see Coligan, *et al.*, 1983), using the organ culture method of Wellings and Jentoft (1972). An organ culture system was prefered for several reasons; (a) gross tissue architecture and cell-cell, cell-substrate interactions are maintained; (b) findings could be related to the relevant clinical data for individual patients; (c) a wide range of breast tissue, both benign and malignant, was available thereby providing opportunities to compare carcinomas of different types and grades with

non-malignant breast tissue. Similar approaches using cell lines (Schwartz, *et al.*, 1986) although useful would have none of the advantages listed above. A classical soft gel approach combined with fluorography was used to identify differences in glycosylation which were further analysed by Western lectin/immuno-blotting techniques. The use of lectins (Boyd, 1963), sugar-binding molecules of non-immune origin, in Western blotting is well documented (Glass, *et al.*, 1981) and of particular importance in studying carbohydrate structure, since they have a remarkable sugar specificity (Sharon and Lis, 1972). Some lectins show a strong specificity for certain anomeric forms such as those which bind to ABO blood group antigens (Watkins, 1980). Many lectins will only react with terminal non-reducing sugars (e.g. peanut agglutinin) whereas others will bind to monosaccharides located both internal or external to the glycan chain (Gallagher, 1984). The lectins used in this study were: the L-fucosyl-binding *Ulex europaeus I* (UEA-I); the N-acetylglucosamine-binding wheat germ agglutinin (WGA), (Bhavanandan and Katlic, 1979); and the terminal galactose-disaccharide-binding *Arachis hypogaea* (PNA or peanut agglutinin), (Pereira, *et al.*, 1976). Antibodies were also used in Western blotting either to identify known antigenic determinants or to highlight novel components. The antibodies used (HMFG1 and HMFG2), identify epitopes present on the MFGM glycoproteins which are of similar size to that of the high molecular weight group of glycoproteins

identified and described in this chapter. Subsequent studies employed other antibodies directed against different glycoproteins (see chapter 4).

The use of peptide mapping to determine the identity or non-identity of the protein core structures of the four high molecular weight glycoproteins was hampered by difficulties in obtaining sufficient quantities of purified glycoprotein. For this reason a number of techniques were attempted in order to overcome this problem. Nevertheless some peptide mapping data was derived from glycoproteins removed from fixed, stained, dried and fluorographed gels.

The molecular weight determination of any glycoprotein separated under reducing conditions in SDS-PAGE, is recognized to yield an abnormally high molecular weight. Errors are diminished with an increased acrylamide concentration, but this unfortunately restricts the adequate separation of high molecular weight components (Segrest, *et al.*, 1971; Leach, *et al.*, 1980). The identification therefore, of specific molecular weights of glycoproteins identified and discussed in this and subsequent chapters, is always approximate.

This chapter is therefore devoted to the analysis of the labelled glycoproteins released from human breast tissue during organ culture, detailing the various methods employed.

This work formed the basis of a publication (Rye & Walker, 1989) and was the subject of part of a

lecture given to the 158th meeting of the Pathological Society of Great Britain and Ireland, (Appendix G).

2.1. The Samples.

2.1.1. Tissue collection.

Human female breast tissue was obtained immediately after surgery at Leicester Royal Infirmary and Glenfield General Hospital Leicester. All samples for analysis were taken by a qualified pathologist (Dr. R.A. Walker). Approximately 1g of tissue from each specimen was placed into 10ml of Eagles Minimal Essential Medium containing 200U/ml penicillin and 200ug/ml streptomycin and kept at 4°C until prepared for organ culture. Tissue was always processed within 60 minutes of receipt. Parallel slices of tissue, (20x15x5mm), were fixed in 4% formaldehyde in saline for 24hrs. Blocks of tissue (10x5x3mm) from each case were frozen in liquid nitrogen onto OCT/cork and stored in the gaseous phase at approximately -140°C

until required for frozen section histology.

The total number of cases collected were;

8 Benign breast disease

5 Fibroadenomas

28 Carcinomas

2.1.2. Tissue processing.

Fixed tissues were dehydrated, cleared and infiltrated by paraffin wax, using a standard Histokinette tissue processor, and embedded in paraffin wax.

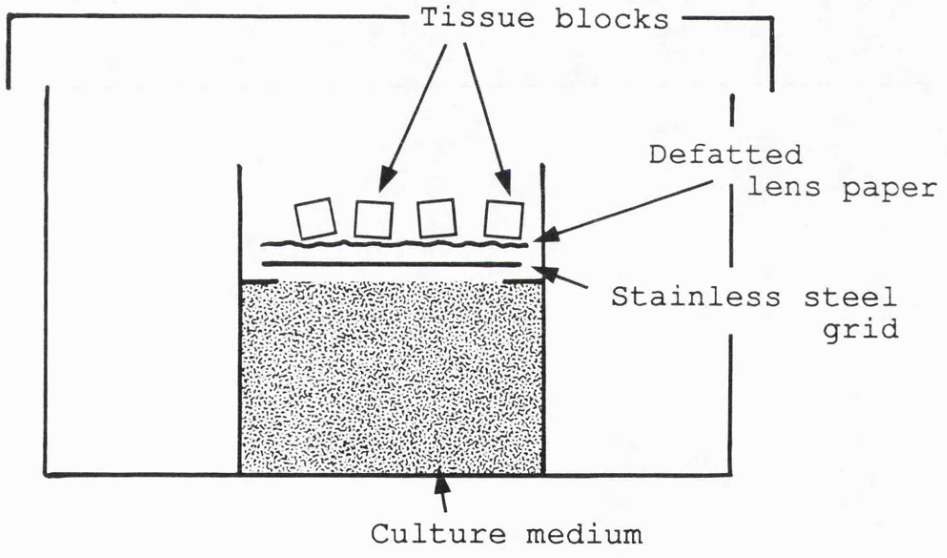
For all samples sections were cut at 4um, dewaxed, rehydrated and stained with haematoxylin and eosin. Most of this work was done by the technical staff of the University of Leicester department of Pathology.

2.1.3. Histological assessment and patient data.

The tissue sections were examined by Dr. R.A. Walker and classified using WHO criteria. Assessment was made of the degree of differentiation of carcinomas using a modification of the Bloom and Richardson grading criteria (Elston, *et al.*, 1982; Elston, 1987). Information was available about tumour size, lymph node status (for 18 cases of patients with carcinomas) and menopausal status.

2.2. Organ Culture.

Figure 2.1. Organ culture system after Wellings and Jentoft.



2.2.1. Procedures and conditions.

All procedures were carried out in a Class II laminar flow cabinet using aseptic technique. Fat and necrotic tissue was removed and the tumour specimens were sliced into 1mm cubes on sheets of dental wax using opposing sterile skin graft blades. A small quantity of this diced tissue was processed for histology (see section 2.1.2.) to check the amount of mechanical damage caused by the cutting. Approximately 100-130mg wet weight of tissue was placed onto acetone washed lens tissue supported by stainless steel grids in 60x15mm organ culture dishes (figure 2.1.), after Wellings and Jentoft, (1972). Dulbecco's Modified Eagles Medium (DMEM) with L-glutamine and 100U/ml penicillin and 100ug/ml streptomycin was used throughout as the culture medium of choice. After a 2hr pre-incubation the medium was replaced with fresh pre-warmed DMEM with penicillin and streptomycin, and 20uCi/ml of [^{3}H] fucose (specific activity 70Ci/mmol), [^{3}H] galactose (specific activity 31Ci/mmol), [^{3}H] glucosamine hydrochloride (specific activity 30Ci/mmol) or [^{3}H] leucine (specific activity 140Ci/mmol) added. Organ cultures were then incubated in an atmosphere of 95% air, 5% CO_2 at 37°C for 18 and 48hrs. After incubation the medium was collected and 1ul of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) per 1ml of medium was added to each sample to a final concentration of 1mMol/l. Prior to storage at -20°C the samples were centrifuged at 13000xg for 10 minutes. The concentration and activity

of the PMSF (James, 1978) was regarded to be sufficient to inhibit protease action when the sample was thawed. The cultured tissue from the control dishes were fixed and processed as previously described (see section 2.1.2.). Haematoxylin and eosin stained sections were assessed for tissue viability. Radiolabelled tissue samples were frozen at -70°C until required for further study (section 6.1.2. and 6.2.1.).

Before routinely using the organ culture method described above, several variations of the system were assessed for their general effect on histological preservation. A submerged method, where tissue explants are fully immersed in culture medium, was tried using both benign and malignant breast tissue and compared with the raft method. A further comparison of these two methods was done using a higher atmospheric concentration of oxygen by incubating submerged cultures in the presence of 95% O_2, 5% CO_2, (see also section 2.6.1.).

Variations in the composition of the culture medium did not appear to affect the tissue morphology. Three types of culture medium were tried with and without a glucose supplement, DMEM, Hams F12, and Medium 199. DMEM the culture medium of choice, is well suited for supporting the growth of a broad spectrum of mammalian cell lines. It was also the cheapest medium of those tested.

2.3. SDS-Polyacrylamide Gel Electrophoresis.

2.3.1. Sample dialysis and concentration.

Two methods were used:

(a). Medium from each incubation was dialysed first against 5 litres of ultra-pure water for 3-4hrs with agitation. Further dialysis with a fresh change of water was continued overnight and all steps were done at 4°C.

Dialysis tubing was pre-treated by boiling for 10min in a 2% solution of sodium carbonate containing 10mM EDTA. The tubing was rinsed thoroughly in ultra-pure water and boiled again for 10min in 2% sodium carbonate with 1mM EDTA. Treated dialysis tubing was stored in this solution at 4°C. Prior to use each length of tubing was rinsed with ultra-pure water.

Concentration of the samples was achieved by the osmotic removal of water through the dialysis membrane. The swollen dialysis bags were placed in a tray containing dry polyethylene glycol for 1-2hrs at 4°C. Recovery of the samples included two rinses of ultra-pure water resulting in a final volume of approximately 0.5ml. A further aliquot of PMSF was added to a final concentration of 1mmol/l before processing for SDS-PAGE.

(b). Medium from the incubations were simultaneously concentrated and desalted in commercially available centrifugal microconcentrators following the manufactors instructions. Addition of PMSF was the

same as described above. This later became the method of choice because of its simplicity, speed and minimal membrane/filter adsorption.

Other methods of protein concentration were considered inappropriate for the type and number of samples involved. Concentration by precipitation with either acetone or ammonium sulphate, although widely used, is unsuitable for protein solutions of 1mg/ml or less (Scopes, 1987). Since the total protein concentrations found in the culture medium were of this same order of magnitude this technique was not used.

Lyophilization of protein samples to remove water is also a very useful method of concentrating protein. However it must be noted that protein modification such as deamination of asparigine and glutamine residues or oxidation of cysteine residues has been known to occur when using this technique (O'Farrell, 1975; Dunn and Burghes, 1986).

Another technique of protein concentration using similar principles to the method of choice, involves the use of dry gel filtration particles, e.g., G-25 Sephadex beads. These dry beads exclude protein and soak up water rapidly. They can easily be removed from the sample by centrifugation. However, since it is not possible to remove all protein from the surface of the beads without extensive washing (and subsequent re-dilution), this method is only suitable for situations where protein loss is not critical.

2.3.2. Protein determination.

Total protein measurements were done using the rapid Coomassie Brilliant Blue based reagent of Bradford (1976). Coomassie Brilliant Blue G-250 (Sigma Chemicals Co. Ltd., Dorset, UK), 100mg, was dissolved in 50ml 95% ethanol. After thorough mixing, 100ml 85% (w/v) orthophosphoric acid (Fisons, Leicestershire, UK) was added and diluted to a final volume of 1l with ultra pure water. After mixing, the solution was filtered to remove any insoluble particulate material, invariably contaminants found in the Coomassie dye. During its shelf life it was sometimes found necessary to refilter the reagent. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.75% (w/v) ethanol, and 8.5% (w/v) orthophosphoric acid. Individual batches of protein reagent were checked for consistency by scanning aliquots of the reagent over the absorbance range 400-700nm using a Unicam SP1700 Spectrophotometer. Aliquots containing no protein or 100ug bovine serum albumin (BSA) were scanned.

The protein assay was prepared as follows: stock protein solution containing 1mg/ml BSA (Sigma Chemicals Co. Ltd.) in ultra pure water were stored in 1ml aliquots at -20°C. Appropriate volumes of this stock solution were added to each of six test tubes (12x150mm) to give the standard curve protein concentrations of 0 (blank), 10, 25, 50, 75 and 100ug/100ul. These standards were always done in duplicate. Volumes were made up to 100ul with ultra

pure water. Unknown protein samples were also prepared using 100ul of sample. To each of these tubes, 5ml of protein reagent was added and mixed by vortexing. Tubes were left to stand at room temperature for 5min before a final mixing. Aliquots from each tube were measured at absorbance 595nm and the weight of the protein plotted against the corresponding absorbance. The resultant standard curve was used to determine the amount of protein in the samples.

2.3.3. Gel preparation.

The SDS-discontinuous buffer system based on the method of Laemmli (1970) was used. Because of the toxicity of acrylamide and the need to avoid skin protein artifacts (Ochs, 1983) gloves were worn throughout. A vertical tank system was used, based on the Studier-type slab gel apparatus (Studier, 1973), which was built by the University workshops. Apparatus for preparing linear concentration gradient gels was designed by the author (see Appendix A) and also built by the University workshops. Electrophoresis plates (20x20cm) and gaskets (1.5mm thick) were obtained through a commercial source. All reagents were of very high purity specifically for electrophoresis.

Stock solutions were prepared as follows:
(i) Acrylamide-bisacrylamide; this was prepared by dissolving 30g acrylamide and 0.8g N,N-methylenebisacrylamide in a final volume of 100ml ultra-pure water, filtered through a Whatman No.1 filter paper and stored in a dark bottle at 4°C. This

solution was prepared fresh every six weeks.

Mid-point through the period of research a supplier of liquid acrylamide-bisacrylamide mixtures was found. The use of these chemicals for the remaining period of research was preferred since it avoided the extremely hazardous manipulations of weighing the dry chemicals. The acrylamide was supplied as a 40% solution in distilled, deionized water and the crosslinker, N,N-methylenebisacrylamide as a 2% solution. The following formulae were used to calculate the volumes of reagent needed to reproduce the concentrations previously used with the dry chemicals;

Va = Volume of acrylamide to be used (ml)

Vb = Volume of bisacrylamide to be used (ml)

Vt = Total volume of gel casting solution (ml)

T = Gel concentration (%)

C = Amount of crosslinker as a percentage of total acrylamide (%)

$$Va = \frac{T(100-C)Vt}{4000}$$

$$Vb = \frac{T\ C\ Vt}{200}$$

(ii) Ammonium persulphate; 0.15g of ammonium persulphate was dissolved in 10ml ultra-pure water.

Table 2.1. Table showing gel compositions using acrylamide powder (upper section) and commercial liquid acrylamide/bisacrylamide preparations (lower section). Modified from Hames & Rickwood 1981.

STOCK SOLUTION	STACKING GEL COMPOSITON (ml)	RESOLVING GEL COMPOSITION (ml)				
		20.0%	15.0%	10.0%	7.5%	5.0%
Acrylamide-bis (30:0.8)	2.5	20.0	15.0	10.0	7.5	5.0
Stacking gel buffer	5.0	-	-	-	-	-
Resolving gel buffer	-	3.75	3.75	3.75	3.75	3.75
10% SDS	0.2	0.3	0.3	0.3	0.3	0.3
1.5% ammonium persulphate	-	1.5	1.5	1.5	1.5	1.5
0.004% riboflavin	2.5	-	-	-	-	-
Ultra pure water	9.8	4.45	9.45	14.45	16.95	19.45
TEMED	0.015	0.015	0.015	0.015	0.015	0.015
Acrylagel (40%)	1.83	14.61	10.96	7.31	5.47	3.65
Bisacrylagel (2%)	0.98	7.80	5.85	3.90	2.93	1.95
Stacking gel buffer	5.0	-	-	-	-	-
Resolving gel buffer	-	3.75	3.75	3.75	3.75	3.75
10% SDS	0.2	0.3	0.3	0.3	0.3	0.3
1.5% ammonium persulphate	-	1.5	1.5	1.5	1.5	1.5
0.004% riboflavin	2.5	-	-	-	-	-
Ultra pure water	9.49	2.04	7.64	13.24	16.05	18.85
TEMED	0.015	0.015	0.015	0.015	0.015	0.015

This solution is labile and was made fresh prior to each use.

(iii) Electrophoresis buffers; the SDS-discontinous buffer system of Laemmli (1970) was used. *Stacking gel* buffer solution (0.5M Tris-HCl, pH 6.8) was made by dissolving 6.0g Tris in 40ml ultra-pure water, titrated to pH 6.8 with 1M HCl (about 48ml) and made up to 100ml final volume with ultra-pure water. The solution was filtered through Whatman No.1 filter paper and stored at 4°C. *Resolving gel* buffer stock solution (3.0M Tris-HCl, pH 8.8) was made by dissolving 36.3g Tris in 48ml 1M HCl and brought to 100ml final volume with ultra-pure water. The solution was filtered through Whatman No.1 filter paper and stored at 4°C. *Reservoir* buffer stock solution (0.25M Tris, 1.92M glycine, 1% SDS, pH 8.3) was made by dissolving 30.3g Tris, 144g glycine and 10g SDS in 1l (final volume) ultra-pure water. The solution was checked for correct pH and stored at 4°C. *Sample* buffer was prepared as a 20ml solution containing 3.2ml stacking buffer, 2.0ml b-mercaptoethanol, 0.02mg/ml bromophenol blue and 4g sucrose. This solution was mixed for 5 minutes at room temperature before adding 5ml of 10% SDS. The solution was stored at 4°C.

(iv) Riboflavin; a 0.004% w/v solution was prepared by dissolving 4mg riboflavin in 100ml final volume ultra-pure water. The solution was stored at 4°C in a dark bottle.

(v) SDS; a 10% w/v solution was prepared by dissolving

10g SDS in 100ml final volume ultra-pure water at room temperature. The SDS used was of high purity since many electrophoretic artifacts have been attributed to SDS of poor quality (Swaney, *et al.*, 1974).
(vi) TEMED; this solution was used as supplied and stored at 4°C in a dark bottle.

For single concentration gels the recipes in table 2.1 were used. The volumes described are for the preparation of two identical slab gels. Gel mixtures were degassed for 20 minutes on ice using a rotary evaporator. The appropriate volume of TEMED was added with gentle but thorough mixing and the gel solution poured into the glass plate sandwich to a height of 110mm. The acrylamide mixture was then carefully overlayed with 2-3ml of butanol saturated water and left to polymerize. After 2hrs the overlay was removed, the top of the resolving gel rinsed and overlayed with 2-3ml of 0.375M Tris-HCl (pH8.8). The gels were stored overnight at 4°C prior to adding the stacking gel.

For preparing linear concentration gradients the gel composition was essentially the same as shown in table 2.1. For example a gradient from 5%-20% was achieved by preparing 30ml of 20% gel (table 2.1.) with the addition of 4.5g sucrose. The same volume of 5% gel was prepared but with the extra addition of 2.5ml ultra pure water. The two solutions were seperately degassed and two identical gradient gels were polymerized using the gradient pouring apparatus (appendix A).

Stacking gels for both single concentration and gradient gels were prepared as shown in table 2.3.1. Riboflavin was used as the catalyst in the photopolymerization of the stacking gel. This method of polymerization was chosen since it avoided a pre-electrophoresis step (which is not advisable with a discontinuous gel system) to remove an otherwise excessive quantity of persulphate ions. It had the added advantage that polymerization would only begin when the gel mixture was illuminated. After degassing, 5ml of the gel mixture was used to rinse the top of the resolving gel the remaining volume was used for the stacking gel. The stacking gel was then illuminated for 45 minutes after which the comb was removed and overlayed with 0.125M Tris-HCl (pH 6.8). The gel was allowed to stand for a further 30 minutes prior to use. A variety of combs were used although a 15 well comb producing a well of 6x25mm was found to be the optimum size. In these experiments polymerization was achieved using two 15W tropical daylight tubes removed from an X-ray viewing box.

2.3.4. Sample application.

Equal volumes of sample and sample buffer were added to small eppendorfs (0.5ml capacity) and placed in a preheated hot-block at 100°C for 5 minutes. Samples were removed, allowed to cool and then centrifuged 6000xg for 5 minutes to remove insoluble material. Samples were then ready to apply to the gel. For each specimen samples containing 100ug total

protein were applied.

2.3.5. Running conditions.

The electrophoresis apparatus was connected to a power pack such that negatively charged molecules would migrate down the gel towards the lower reservoir. Electrophoresis began at 40V until the samples had entered the stacking gel. The voltage was then increased to 60V and left at room temperature overnight. The power was switched off when the dye front was about 0.5cm from the base of the resolving gel.

2.3.6. Molecular weight determination.

Electrophoretic runs were calibrated with a molecular weight standard mixture containing myosin (205kDa), b-galactosidase (116kDa), phosphorylase b (97.4kDa), bovine serum albumin (66kDa), egg albumin (45kDa) and carbonic anhydrase (29kDa). A standard curve was drawn for each gel by plotting $\log_{10}$ molecular weight against the distance migrated (in mm) by the molecular weight markers.

The behaviour of certain types of molecules in this system neccesitates that molecular weight determinations can only be estimated. Many glycoproteins behave anomalously in SDS-PAGE despite the excess of detergent and disulphide reducing agents. It is known that glycoproteins with a high carbohydrate component migrate at slower rates than would be expected for their size (Segrest, *et al.*,

1971) and this was shown to be due to their reduced binding of SDS. This would show these molecules to have an artifactually high molecular weight. However the abnormal mobilities of glycoproteins in SDS-PAGE is markedly reduced with increased acrylamide concentration (Leach, *et al.*, 1980) and for this reason in most cases the lowest concentration used was a 10% gel. The use of 7.5% gels were later used in some cases in order to expand the high molecular weight regions.

2.3.7. Protein staining.

The staining of proteins in SDS-PAGE was mainly done using the conventional methanol-based Coomassie Brilliant Blue stain. Silver stain techniques were also employed and these are described below in section 2.3.8. Coomassie Blue R250 was dissolved in water/methanol/glacial acetic acid (5:5:2, v/v) to give a 0.1%w/v solution of dye. The solution was then filtered to remove insoluble material before use. The resolving gel was stained in a covered glass casserole dish with 500ml of this solution for 1 hour. The gels were destained, in several changes of the same solution without the dye, for about 2 hours at room temperature. From the stained gels the migration distances of the molecular weight standards were measured and a standard curve produced (see section 2.3.6). A photographic record of each gel was made at this stage. Staining of gels for subsequent peptide mapping were stained and destained for a maximum total

time of 1 hour.

2.3.8. Silver staining.

Silver staining of gels prior to fluorography was not performed since the precipitation of silver granules in the staining procedure results in severe quenching of the low energy β-particles produced by the radio-labelled glycoproteins (Van Keuren, *et al.*, 1981).

The method for silver staining polyacrylamide gels was chosen on the basis of speed, simplicity and low background staining. These criteria were satisfied using the method of Merril, *et al.*, (1981). All incubations were carried out in chromic acid cleaned glass dishes and the times stated are for gels of 1.5mm thickness. Stock solutions of oxidizer and silver were made as 10 fold concentrates and stored in dark bottles at 4°C. Developer solution was made fresh and formaldehyde added immediately before use.

Gels to be silver stained were first fixed in 50% methanol, 12% acetic acid for 60 minutes followed by a further 2 x 30 minutes in 10% methanol, 5% acetic acid. The gels were then soaked in a 3.4mM potassium dichromate solution containing 0.0032N nitric acid for 10 minutes. After washing for 3 x 10 minutes in ultra-pure water the gels were placed into a separate dish containing 0.012M silver nitrate solution for 30 minutes, the first 5 minutes being exposed to two 10W full spectrum fluorescent light tubes. The gels were washed briefly (2 minutes) in ultra-pure water before

developing in 0.28M sodium carbonate with 0.2% formaldehyde, until colour development had reached an acceptable density. Development was stopped by incubating in 1% acetic acid for 5 minutes. Stained gels were finally rinsed in ultra-pure water and scanned using a laser densitometer. The gels were stored in heat sealed polythene bags at 4°C.

2.3.9. Fluorography and densitometry.

After Coomassie Blue staining the gel was rinsed briefly in ultra-pure water to remove excess acetic acid. The gel was incubated at room temperature in 1M sodium salicylate for 30 minutes (Chamberlain, 1979) and then dried onto a filter paper backing in a vacuum slab gel drier for 3hrs at 60°C. Concentration gradient gels, which are more prone to cracking whilst drying, were soaked in a solution of 30% methanol containing 3% glycerol for 30 minutes before drying. The dried gels were then exposed to pre-flashed X-ray film at -70°C for 3 weeks.

The resultant fluorograms were scanned using a scanning laser densitometer.

2.4. Protein Blotting.

2.4.1. Electrotransfer methods.

The protein blotting method used was that of Towbin, *et al.*, (1979) but with some modifications. Gels for blotting were incubated at room temperature

for 30 minutes in a Tris (25mM), glycine (192mM) transfer buffer, (pH 8.3). A sheet of nitrocellulose (NC), pore size 0.45um, was soaked briefly in the same buffer taking care to ensure even wetting. After incubation the gel, supported by several layers of filter paper was placed on the cathode side of a blotting cassette. The pre-wetted NC sheet was carefully placed over the gel making sure not to trap air bubbles as this would impede transfer of material from the gel to the NC. Further layers of filter paper were placed on top of the NC and the cassette closed and placed into the blotting tank with fresh transfer buffer. Blotting was carried out overnight at a 300mA constant current, in a 4°C cold room. Buffer temperature was maintained at about 15°C. After transfer the NC sheet was removed from the cassette and strips containing the molecular weight standards were cut out and stained in 0.2% Amido Black 10B (Bio-Rad) in methanol/acetic acid/water solution (described in section 2.3.7) for 5 minutes and destained. The strips were then rinsed thoroughly in phosphate buffered saline (10mM) to prevent excessive shrinkage of the NC strips (Gershoni & Palade, 1982). As well as having a means of estimating molecular weight these strips also served as a visual indication of effective transfer to the NC. (The remainder of the NC sheet was processed as described in sections 2.4.2 to 2.4.4.). The blotted gel was stained as described in section 2.3.7. to check that effective transfer from the gel had occurred.

This method of transfer was essentially based on that described by Towbin, *et al.*, (1979) but with the omission of 20% methanol from the transfer buffer. Methanol increases the binding capacity of NC by strengthening hydrophobic interactions and it also maintains the geometry of the gel by preventing swelling in the low ionic strength transfer buffer. When methanol is omitted from the buffer gel swelling would normally occur during transfer resulting in the distortion of bands on the NC. To avoid this it was necessary to pre-swell the gel, by soaking in buffer as described earlier, before transfer occurs. Although methanol increases the binding capacity of NC it effectively denatures the proteins in the gel matrix. This fixation severely retards the elution of high molecular weight proteins and long transfer times are necessary to achieve effective elution of material from the gel (Burnette, 1981; Gershoni & Palade, 1982; Nielson, *et al.*, 1982). Furthermore, if methanol is omitted from the transfer buffer the anionic detergent SDS remains bound to the proteins thereby effecting a more efficient transfer of high molecular weight glycoproteins. Nevertheless high blotting times were still required to elute the high molecular weight components.

Since blotting was performed overnight it was necessary as a safety precaution to transfer at a constant current (Gershoni & Palade, 1982). As blotting proceeds electrolytes are eluted from the gel which increase the conductivity and decrease the

resistance of the transfer buffer. This in turn increases the current in the system which leads to a marked rise in buffer temperature. The constant current system used ensured that as blotting progressed the voltage gradually decreased and there was therefore no danger of the system overheating.

2.4.2. Quenching/blocking protein blots.

A variety of methods are used as blocking agents of non-specific sites on nitrocellulose membranes. Some of these blocking agents however are inappropriate when blotting with certain types of ligands. For this reason two types of blocking agents have been used; (a) periodate treated BSA (Glass, *et al.*, 1981), for lectin-blotting and, (b) BSA (Towbin, *et al.*, 1979) for immuno-blotting.

(a).Periodate BSA is prepared by dissolving 4g BSA in 100ml sodium acetate (pH 4.5) and adding 10ml of 100mM periodic acid. This mixture was left at room temperature for 6hrs to inactivate any non-specific lectin-binding activity. The excess periodate was removed by adding 11ml of 100mM glycerol and dialysing against two changes of 10mM phosphate buffered saline, PBS (appendix B), for 3hrs each at 4°C. The solution was diluted to give a BSA concentration of 3%, which was subsequently used at 37°C for 1hr in order to block the nitrocellulose membranes.

(b). 3% BSA in 10mM PBS was used at 37°C for 1hr to block membranes in preparation of using antibodies. Normal serum from the same species as the

secondary antibody was also added to the blocking solution to prevent non-specific binding of immunoglobulin.

A more recent blocking agent, Tween-20 (Batteiger, *et al.*, 1982), was used in later studies at 0.3% v/v at 37°C for 1hr and, although it tended to produce a slightly higher background than BSA, it was the preferred method since it allowed the blot to be stained for protein (0.2% amido black-10B), after it had been probed.

2.4.3. Lectin-blotting.

Subsequent to the blotting and appropriate blocking protocols the blot was incubated for 2hrs at room temperature with one of the peroxidase labelled lectins; *Ulex europeus I* (20ug/ml), *Lotus tetragonolobus* (20ug/ml), wheat germ agglutinin (10ug/ml) and peanut agglutinin (10ug/ml). When using peanut agglutinin it was first necessary to remove terminal sialic acid residues by incubating the blot for 1hr at 37°C with 0.25 Units/ml neuraminidase in acetate buffer (pH 5.4) containing 100mM $CaCl_2$ (see appendix B). After incubation with lectin, the blot was extensively washed in three 20 minute changes of PBS containing 0.05% Tween 20.

Detection of the bound lectin exploited the diaminobenzidine (DAB) hydrogen peroxide reaction. A fresh solution of 0.5mg/ml DAB in PBS was prepared and filtered before each use. Immediately before use hydrogen peroxide was added to a final concentration

of 0.3% and the blot stained at room temperature for 5 minutes.

2.4.4. Immuno-blotting.

Blots were processed as described in section 2.4.2., the serum component in the blocking solution being dependant on the species of the secondary antibody. The blot was incubated for 2hrs at room temperature with the primary antibody, washed extensively in three 20 minute changes of PBS and the appropriate peroxidase labelled secondary antibody (1/50) added for a further 45 minute incubation at room temperature. A second wash period preceeded the visualization with DAB (section 2.4.3.).

Two primary antibodies were used in these experiments and these are listed below showing their titres and the secondary antibody used.

HMFG1	1/20	Rb-Mo
HMFG2	1/20	Rb-Mo

Rb-Mo= Rabbit anti mouse immunoglobulin antisera. (Immunoblotting using a further two monoclonal antibodies is also described in chapter 4).

2.5. Peptide Mapping.

Since the presence or absence of the glycoproteins from any individual tumour could only be determined after fluorography, the subsequent peptide

mapping procedures had to be performed on stained (Phelps, 1984), dried (Perides, *et al.*, 1985) and fluorographed gels (Saris, *et al.*, 1983). This problem combined with the inherent difficulties of working with such a small amount of tumour tissue resulted in a variety of approaches being used with limited success.

2.5.1. Peptide mapping in SDS polyacrylamide gels.

A copy of the fluorogram was made on a sheet of acetate which was used as a guide to cut out the appropriate bands (approx. 2mm x 6mm) on the dried gel. The filter paper backing was carefully teased away and the gel slice rehydrated in 60ul ultra-pure water for 15 minutes at room temperature. The rehydrated gel slice was blotted to remove excess liquid and processed for either enzyme or heat cleavage.

Enzyme cleavage: Two enzymes with different proteolytic specificities were used in the enzymatic cleavage of the glycoproteins; trypsin and the *Staphylococcus aureus* V8 protease, the protocol used was that described by Cleveland *et al.*, (1977). The rehydrated gel slice was soaked for 20 minutes in 0.125M Tris buffer (pH 6.8) containing 0.1% SDS then placed, with the buffer, into the well of a 2.5cm stacking gel on a 20% SDS polyacrylamide gel. The gel slice was overlayed with 15ul of this buffer containing 20% glycerol. To each well was added 10ul of either trypsin or *Staph. aureus* V8 (25ug/ml) in the

Tris buffer containing 10% glycerol and trace bromophenol blue and electrophoresed at 10mA until the bromophenol dye front had migrated well into the stacking gel. Power was switched off for 30 minutes to allow proteolysis to take place and then electrophoresis continued until the dye front reached the bottom of the resolving gel. The gel was finally silver stained as described in section 2.3.8.

Heat cleavage: The rehydrated gel slice was incubated with 100ul Tris buffer (as described above but omitting the glycerol and bromophenol blue) for 60 minutes at 100°C (after Rittenhouse and Marcus (1984), with modifications). After incubation the mixture was allowed to cool to room temperature and then both gel and buffer added to the well of a stacking gel on a 20% polyacrylamide gel. The gel slice was overlayed with 15ul of the Tris buffer containing 20% glycerol and trace bromophenol blue and electrophoresed overnight, until the dye front reached the bottom of the resolving gel. The gel was finally silver stained as described in section 2.3.8.

2.5.2. Peptide mapping in 2-D TLE/TLC.

An alternative protocol for preparing a peptide map was attempted. This involved an extended period of hydrolysis in order to yield smaller peptides, thereby avoiding any difficulties in diffusion from the gel slice. As a result of this, peptide fragments with the anticipated size of 20 residues were generated which could be separated using a two dimensional thin layer

technique involving, Thin Layer Electrophoresis (TLE) and Thin Layer Chromatography (TLC), (Allen, 1986). Before using any of the limited supplies of the glycoprotein 230/250kD the technique was tested with two "control" proteins; galactosidase and phosphorylase. It was anticipated that the results using these two high molecular weight proteins would provide a rough guideline to, (a) the suitability of the technique, and (b) the extent of hydrolysis required for the glycoproteins identified.

The method of generating peptide fragments by heat cleavage, rather than enzyme cleavage, was prefered because the enzyme itself contributed so much to the map as to obscure the final result. The heat cleavage method used was similar to that described by Rittenhouse and Marcus, (1984) but modified to provide a more extensive hydrolysis. To further improve the diffusion of heat cleaved peptides it was found necessary to fragment the dried gel slice prior to the rehydration as described in section 2.5.1. The rehydrated gel fragments were heated at 100°C for 20 minutes in 250ul 0.15N HCl. After heating, 400ul of cold 100mM ammonium hydrogen carbonate was added to the mixture and the heat cleaved peptides left to diffuse from the gel at 4°C overnight. The gel fragments were removed and discarded after being washed with fresh ammonium hydrogen carbonate. The remaining fractions containing the peptides were pooled and lyophilized.

The lyophilized sample was dissolved in 5ul 20%

pyridine and spotted onto a 20cm x 20cm plastic backed cellulose thin-layer plate, allowed to dry and placed on a cooling plate in a horizontal electrophoresis chamber containing the buffer pyridine/acetic acid/acetone/water (20:40:150:790, v/v). Using two strips of Whatman No. 3MM paper soaked in the electrophoresis buffer and placed either side of the application spot, the sample was concentrated at the point where the solvent fronts met. The wicks, also Whatman No. 3MM, were then applied at each end. A glass cover plate was then positioned, resting on the wicks, to prevent excessive evaporation of solvent and maintain a constant atmosphere next to the thin-layer plate. The safety lid was placed on the apparatus and the system was left to equilibrate for 5 minutes before switching on the power. The thin layer was then run at 500 volts for 90 minutes at 20°C. The plate was removed allowed to air dry, turned through 90° and then subjected to TLC in the second dimension using the solvent system, n-butanol/acetic acid/water/pyridine (15:3:12:10, v/v), until the solvent front reached 1cm from the top edge of the plate. The plate was removed and allowed to air dry thoroughly in a fume hood.

Detection of peptides in the 2-D thin layer plate used the reagent ninhydrin in combination with a chlorination reaction (Reindel & Hoppe, 1954). The thin layer plate was sprayed lightly with a solution of 0.2g ninhydrin in 100ml of 95% ethanol/acetic acid (20:1, v/v) and dried using a hot air drier in a fume

hood. Before chlorination the plate was left on the bench for at least 1hr to ensure equilibration to atmospheric humidity and that all solvent had evaporated. The thin layer plate was then exposed to Cl_2 for 10 minutes. Chlorine gas was generated by adding 2-3ml of 12M HCl to 3g of potassium permanganate in a covered beaker. When enough Cl_2 was formed it was decanted into a TLC tank containing the thin layer plate. After exposure the plate was removed and thoroughly aerated in the fume hood (about 45 minutes). The chlorinated peptides were revealed by spraying the plate with a 1% solution of potassium iodide in 1% soluble starch (Rydon & Smith, 1952). This earlier variation of the Rydon & Hoppe method avoids the use of the carcinogen o-toluidine.

2.6. Results.

2.6.1. *Culture conditions.*

Comparison of the protein profile of medium released from breast tissue incubated by both the raft and submerged methods of culture (section 2.2.1.), showed significant differences when analysed by SDS-PAGE and Coomassie Blue staining (figure 2.2.). Moreover when the same media was blotted to nitrocellulose and probed with an anti-cytokeratin antibody to assess the extent of cell breakdown, there was a slightly greater amount of staining for cytokeratins from tissue incubated by the submerged

Figure 2.2. Coomassie blue stained 10-20% linear gradient gel showing differences between proteins released from submerged (A) and raft (B) organ culture for a benign breast lesion.

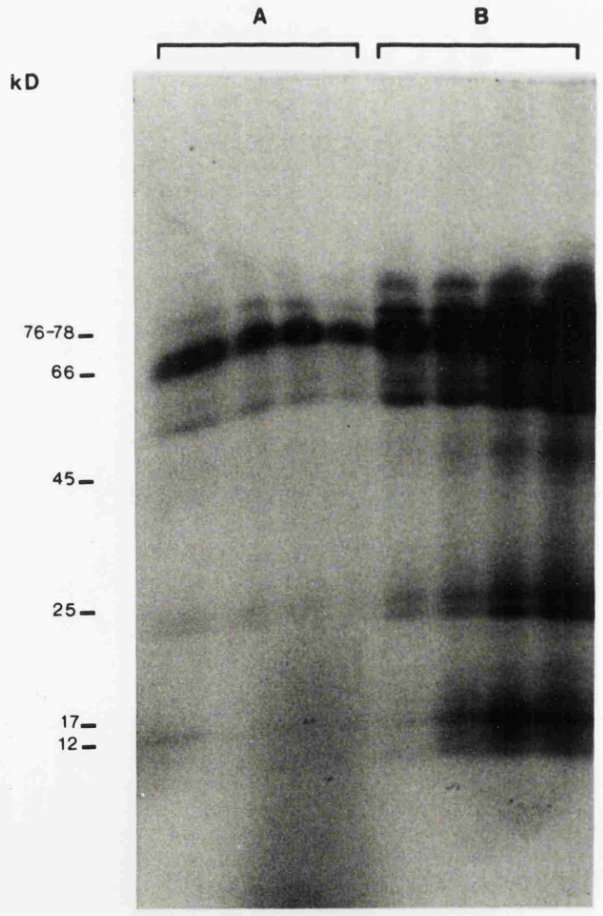


Figure 2.3. Dot blots of medium released from a carcinoma during organ culture labelled with an anti cytokeratin antibody. Raft culture (A) and submerged culture (B).

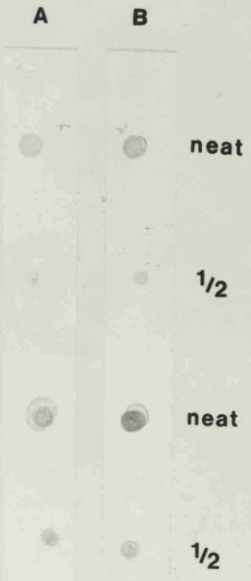


Figure 2.4. Haematoxylin and eosin stained tissue sections of human breast tissue after submerged (A) and raft (B) organ culture.

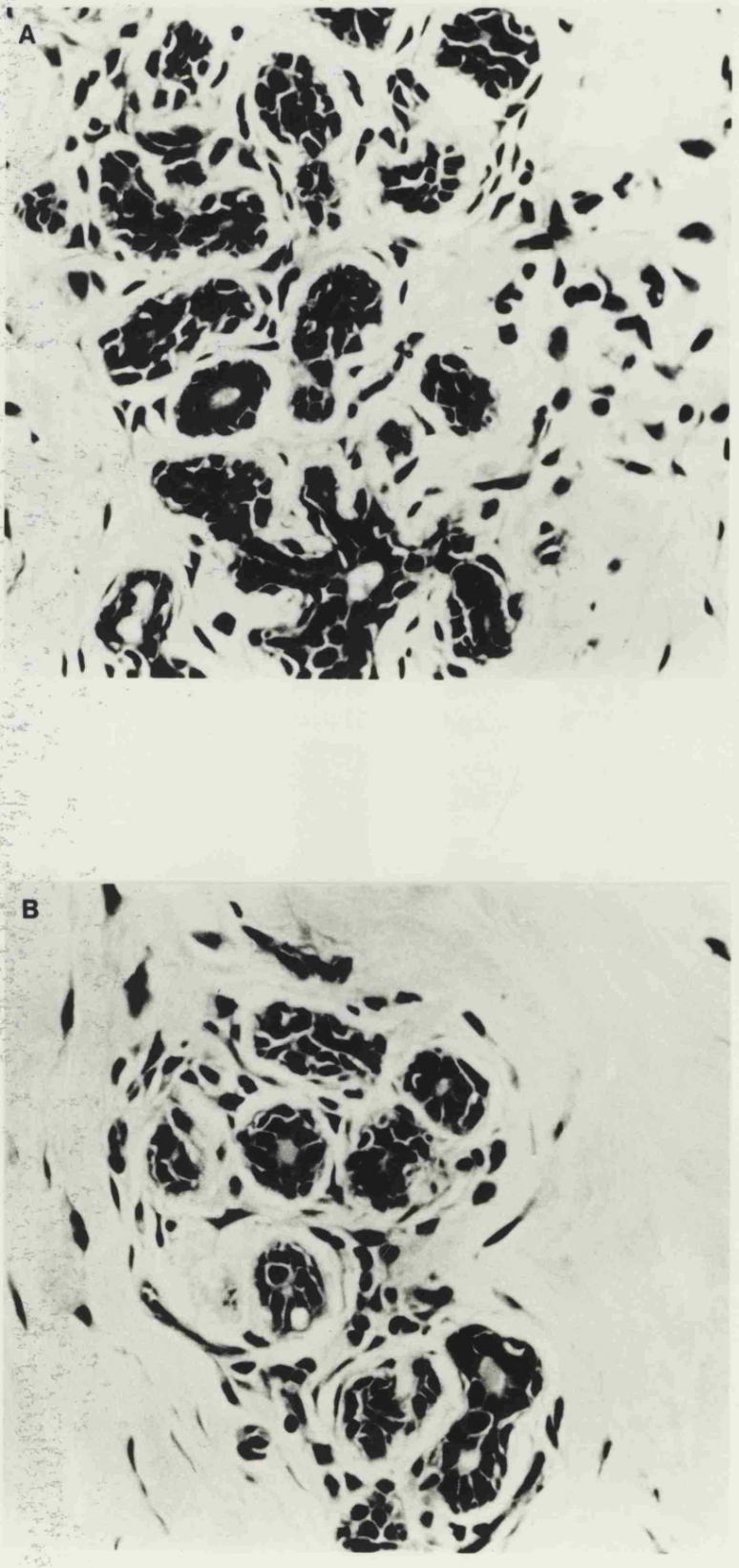


Figure 2.5. Fluorograms showing tritiated products released from moderately hyperplastic benign breast at two incubation times. [3H] Leucine, 18 hours (A) and 48 hours (B); [3H] galactose 18 hours (C) and 48 hours (D); [3H] fucose 18 hours (E) and 48 hours (F).

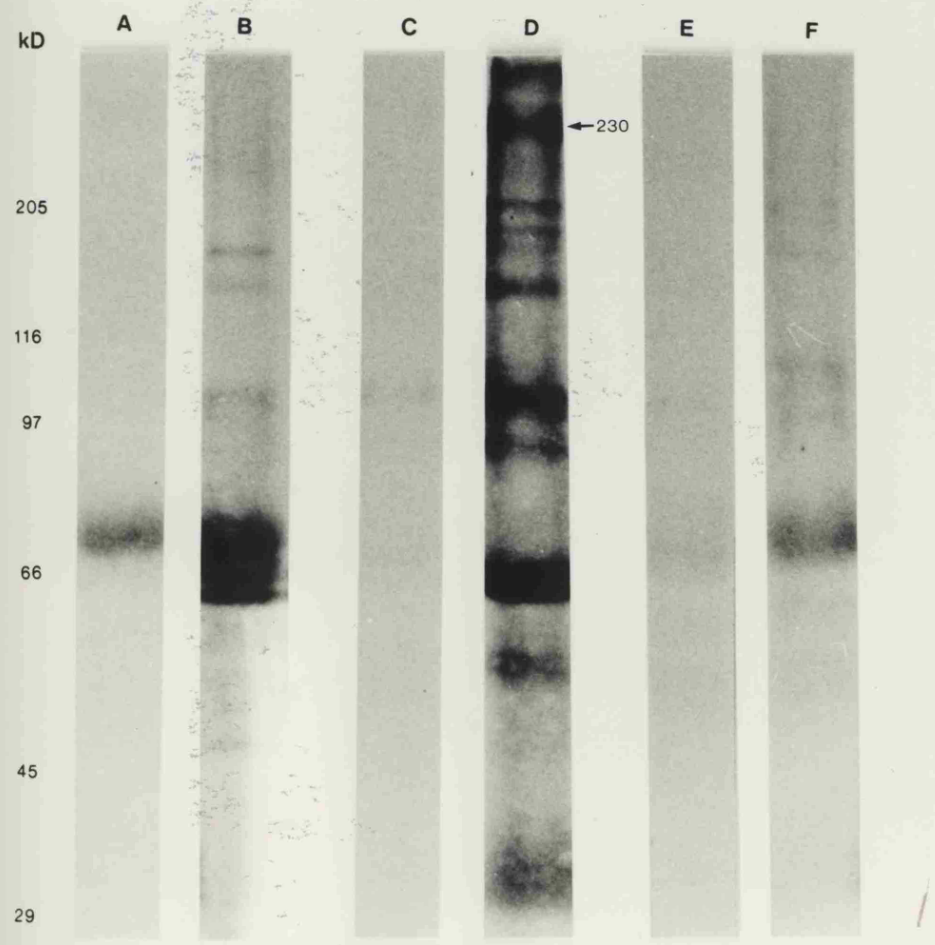


Figure 2.6. Coomassie blue stained 10-20% linear gradient gels showing differences between 18 hour (A) and 48 hour (B) incubations for a ductal carcinoma.

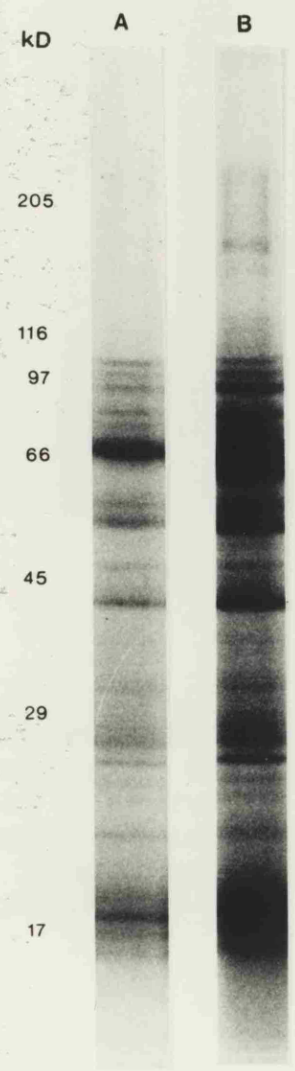


Figure 2.7a. Fluorograms showing glycoproteins released after incorporation of [^{3}H] glucosamine by malignant (A-D) and benign lesions (E-G).

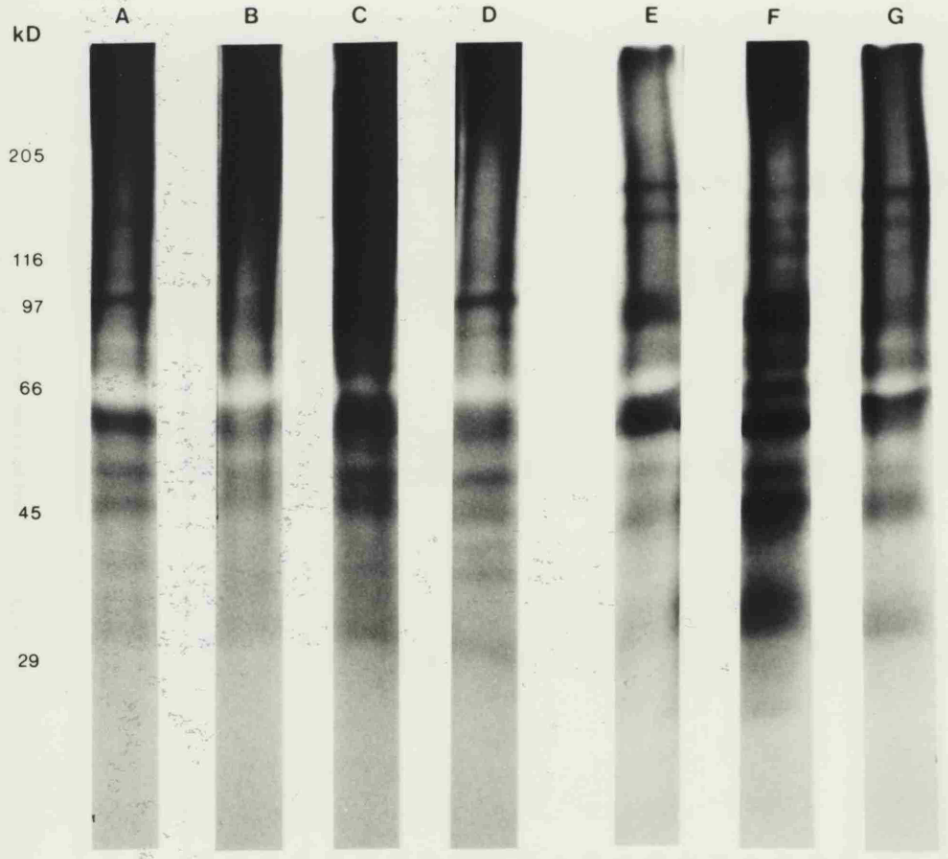


Figure 2.7b. Fluorograms showing glycoproteins released after incorporation of [^{3}H] fucose by a malignant breast lesion (H) and metastatic lymph node (I) from the same patient.

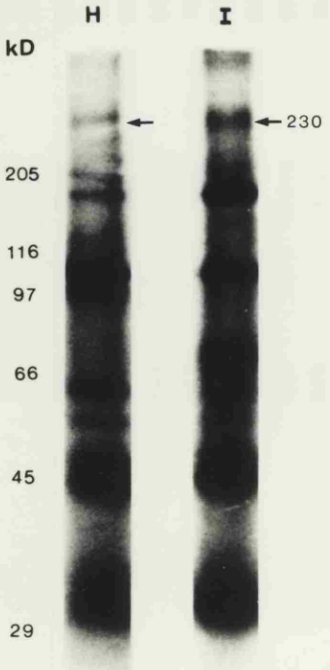


Table 2.2. Patient data and glycoprotein expression in carcinomas.

ID= intraductal, MED= medullary, SEC= secretory, IL= intralobular, POS= positive, NEG= negative, NK= not known, F= fucose, G= galactose. (644*= no fucose data, 670*= no galactose data).

CASE No.	MENOPAUSE STATUS	TUMOUR TYPE	TUMOUR GRADE	NODE STATUS	GLYCOPROTEIN EXPRESSION 210	230	250	280
459	POST	ID	2	POS	+FG	-	-	-
470	POST	ID	3	NK	-	+F	+G	-
472	POST	ID	2	NK	+G	+F	+F	+G
475	POST	ID	3	POS	+FG	+FG	-	-
476	PERI	ID	3	POS	+G	+FG	+G	-
479	PERI	ID	2	NK	-	+F	+G	-
480	POST	ID	3	NK	+FG	-	+G	+FG
502	POST	ID	3	NK	+FG	-	+G	+FG
507	POST	ID	2	POS	+FG	+FG	-	+G
510	PRE	ID	3	POS	+FG	+F	-	+FG
515	POST	ID	2	NK	+F	+FG	-	-
520	PERI	ID	2	NK	-	+FG	+FG	-
522	POST	ID	2	POS	-	+F	-	-
525	POST	SEC	1	POS	ill defined high mwt.			
529	PERI	ID	3	POS	+F	+G	-	+G
535	POST	ID	3	NEG	+FG	-	+FG	+F
543	POST	MED	3	NK	+FG	-	+FG	+F
547	POST	ID	2	NK	+FG	-	+FG	+FG
601	POST	ID	3	NEG	+G	+FG	-	-
644*	POST	ID	2	POS	+G	-	-	+G
647	POST	ID	2	NEG	-	+FG	-	+G
648	POST	ID	3	NEG	-	+G	+FG	+G
651	POST	ID	1/2	NEG	+FG	-	+F	+G
666	POST	IL	2	NK	-	+FG	-	+FG
670*	POST	MED	3	POS	-	+F	-	+F
673	POST	ID	3	POS	-	-	+F	+G
675	POST	IL	2	NEG	+FG	-	-	+FG
697	POST	ID	1	POS	+FG	+FG	-	+G

Table 2.3. Patient data and glycoprotein expression in benign cases. HYP= benign hyperplasia, FAD= fibroadenoma, F= fucose, G= galactose.

CASE No.	MENOPAUSE STATUS	LESION TYPE	GLYCOPROTEIN EXPRESSION 210	230	250	280
449	PRE	HYP	-	-	-	-
455	PRE	FAD	-	-	-	-
458	PRE	HYP	-	-	-	-
497	PRE	HYP	+G			+G
516a	PRE	FAD	-	-	-	-
516b	PRE	FAD	-	-	-	-
532	PRE	FAD	+G	-	-	-
533	PERI	HYP	+G	-	-	-
540	PERI	FAD	-	-	-	-
592	PERI	HYP	+G	-	+FG	+G
646	PRE	HYP	+G	-	-	+G
649	PRE	HYP	-	-	+G	+G
665	PRE	HYP	+FG	-	+G	+G

Figure 2.8. Densitometric profiles of fluorograms of [^{3}H]fucose labelled glycoproteins. 10% SDS-PAGE. (a)Benign hyperplastic breast. (b)Poorly differentiated carcinoma.

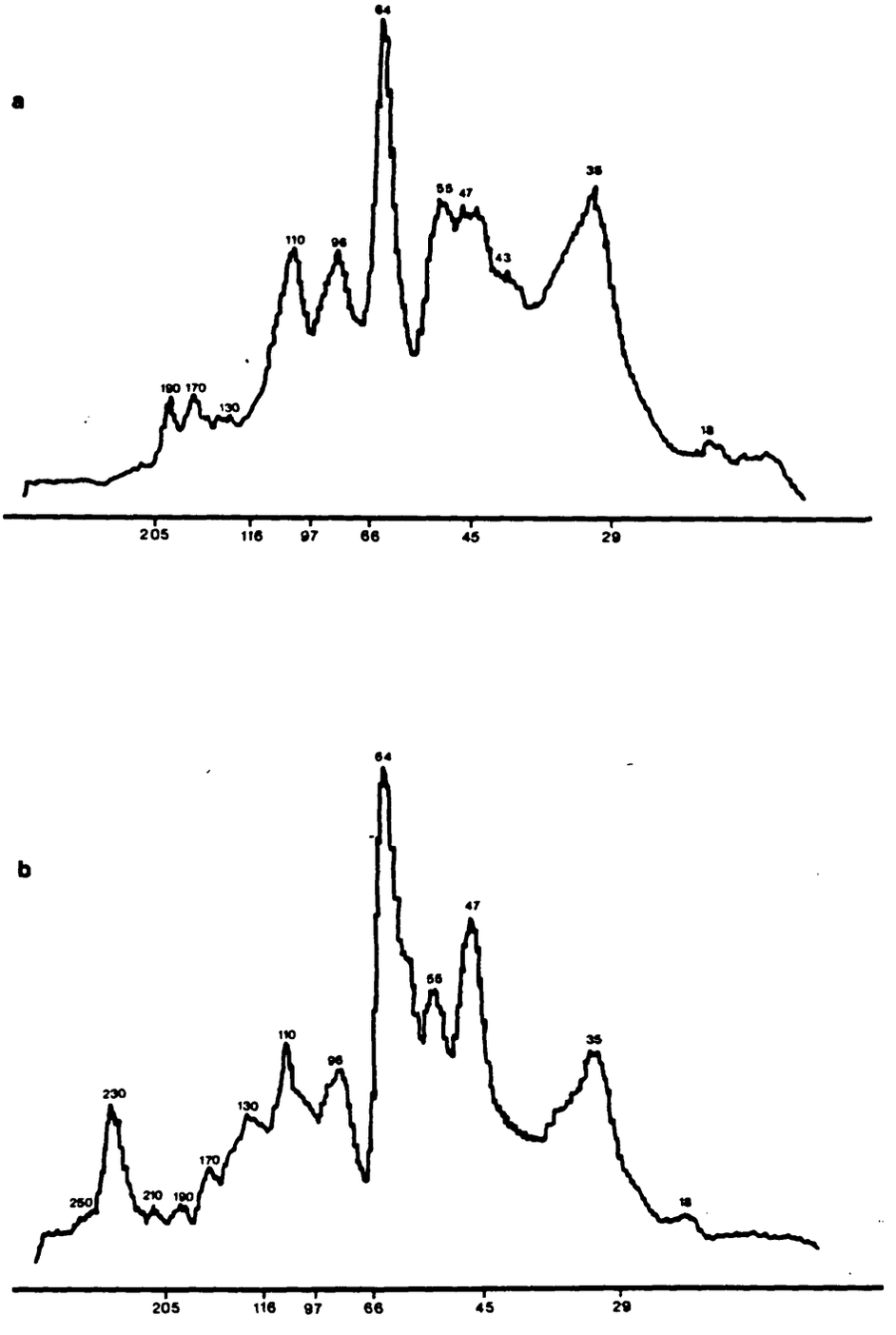
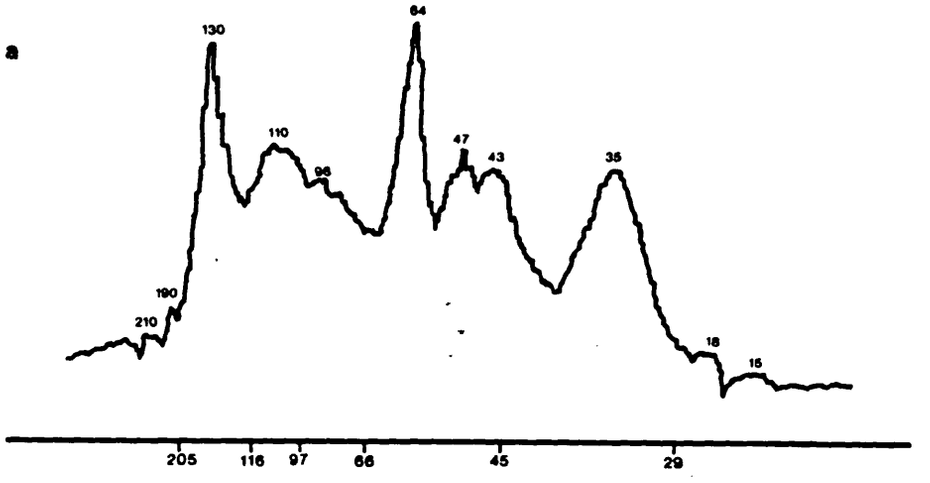


Figure 2.9. Densitometric profiles of fluorograms of [^{3}H]galactose labelled glycoproteins. (a)Fibroadenoma. (b)Moderately differentiated carcinoma.



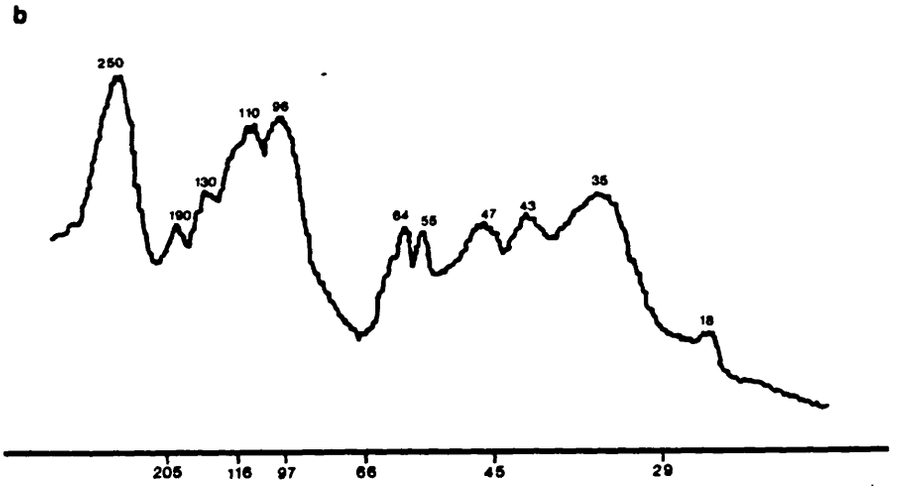


Figure 2.10. Densitometric profiles of fluorograms of labelled glycoproteins released from benign mildly hyperplastic breast. 10-20% SDS-PAGE. (a) [^{3}H]Galactose. (b) [^{3}H]Fucose..

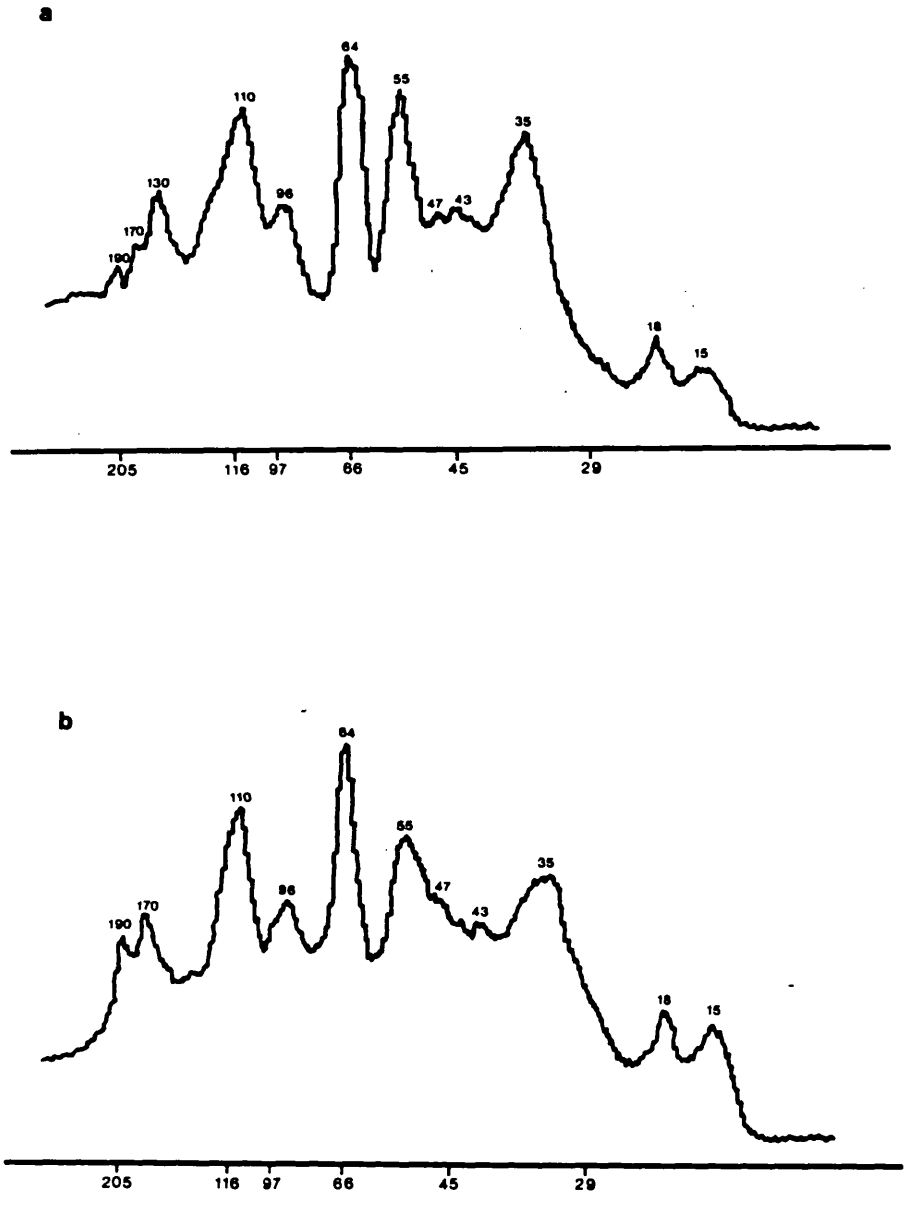
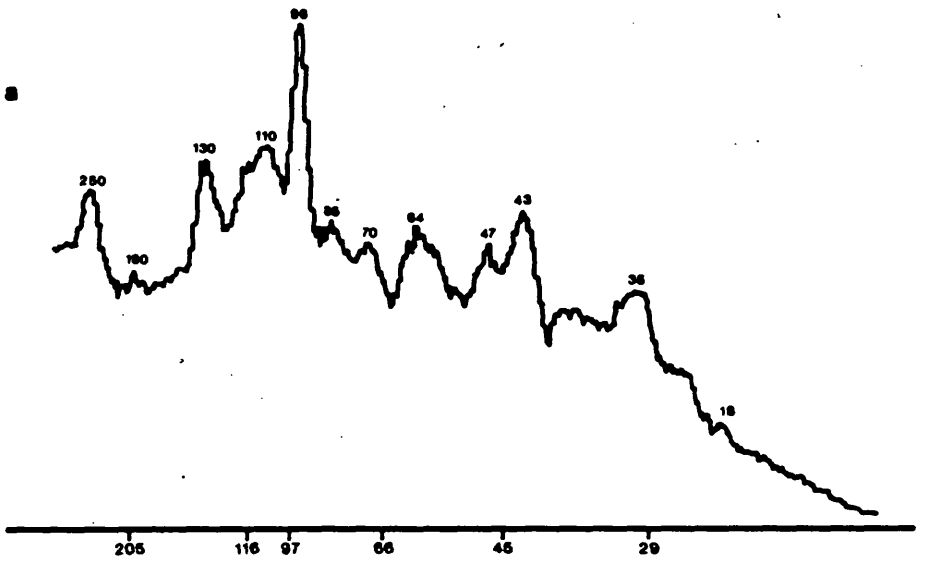


Figure 2.11. Densitometric profiles of fluorograms of labelled glycoproteins released from a moderately differentiated carcinoma. 10-20% SDS-PAGE.
(a) [^{3}H]Galactose. (b) [^{3}H]Fucose.



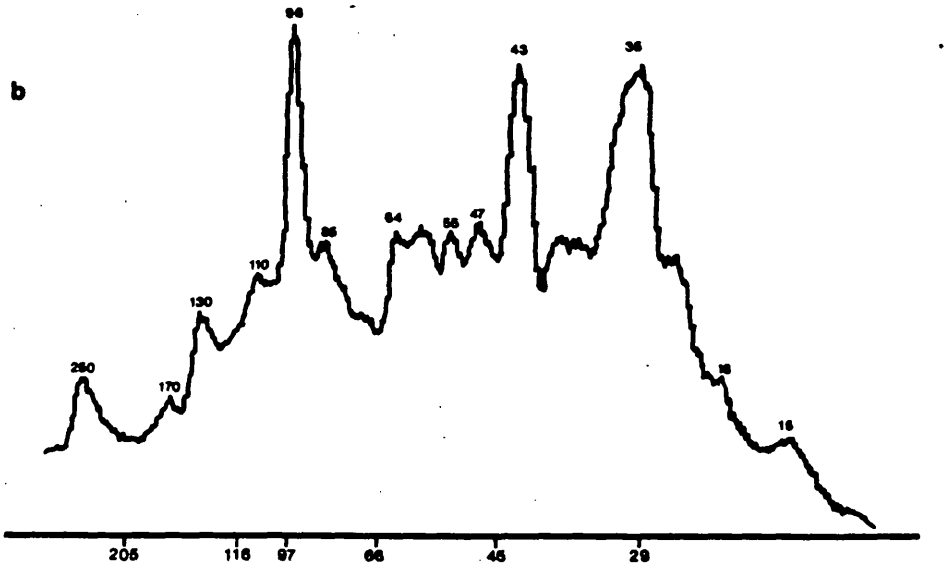


Figure 2.12. Western blot of proteins released from malignant, (lanes A,D and G), and benign, (lanes B,C,E,F and H), cultured breast tissue, showing binding of WGA (lanes A-C), PNA (lanes D-F) and UEA (lanes G and H).

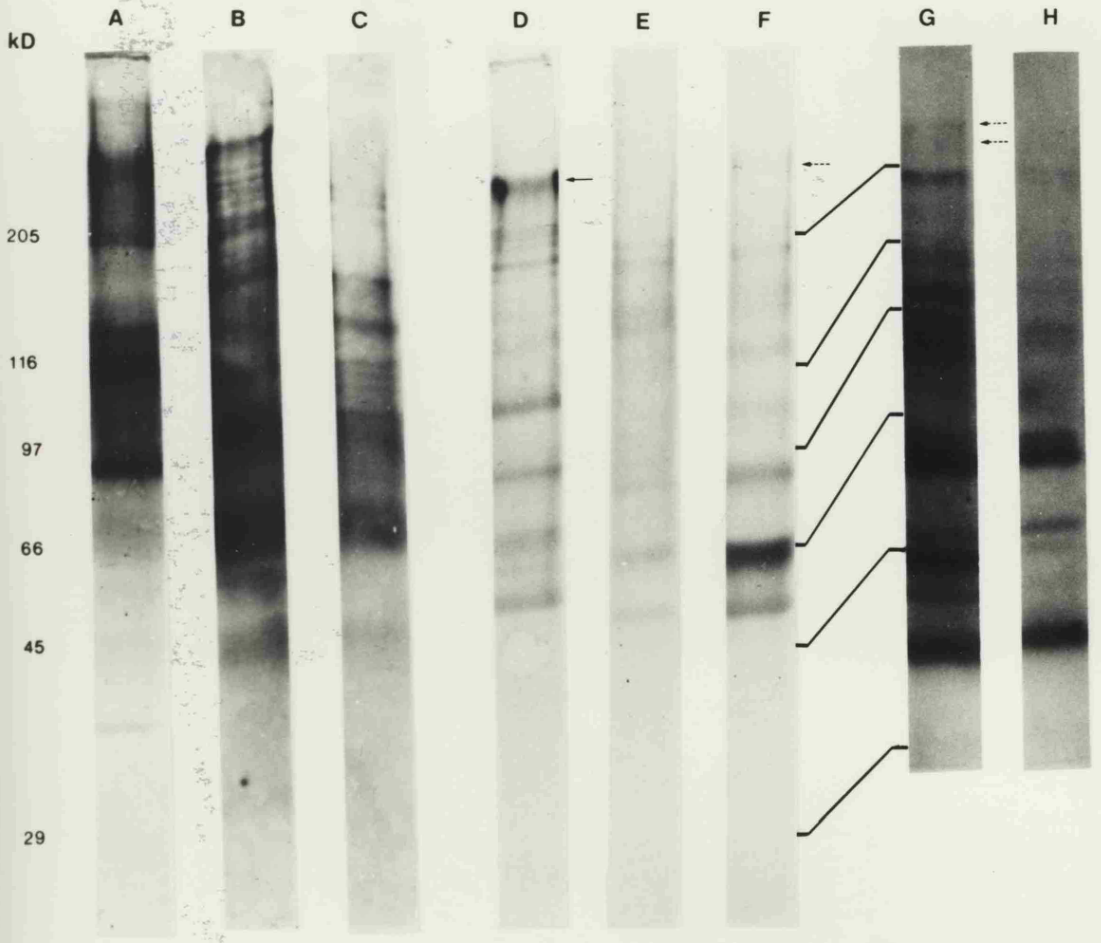


Figure 2.13. Western blot of proteins released from benign cultured breast showing binding of PNA lectin with neuraminidase pre-treatment (A) and without (B).

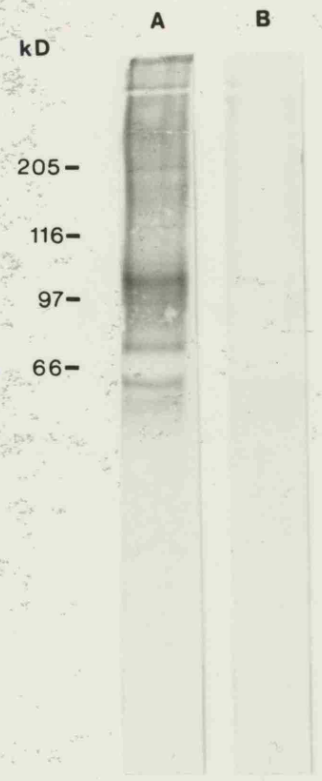


Figure 2.14. Western blot of proteins released from malignant (A,D,E,F,I and J) and benign (B,C,G and H) cultured breast, showing binding of HMFG I (A-E) and HMFG II (F-J).

Figure 2.15. Diagramatic presentation of silver stained 20% SDS-PAGE peptide map of glycoproteins (gp) derived from fixed, stained and dried acrylamide gels.
A,fucose labelled gp 230kD; B,galactose labelled gp 230kD; C,fucose labelled gp 280kD; D, galactose labelled gp 280kD; E, galactose labelled gp 250kD; F,fucose labelled gp 230kD; G,galactose labelled gp 230kD.

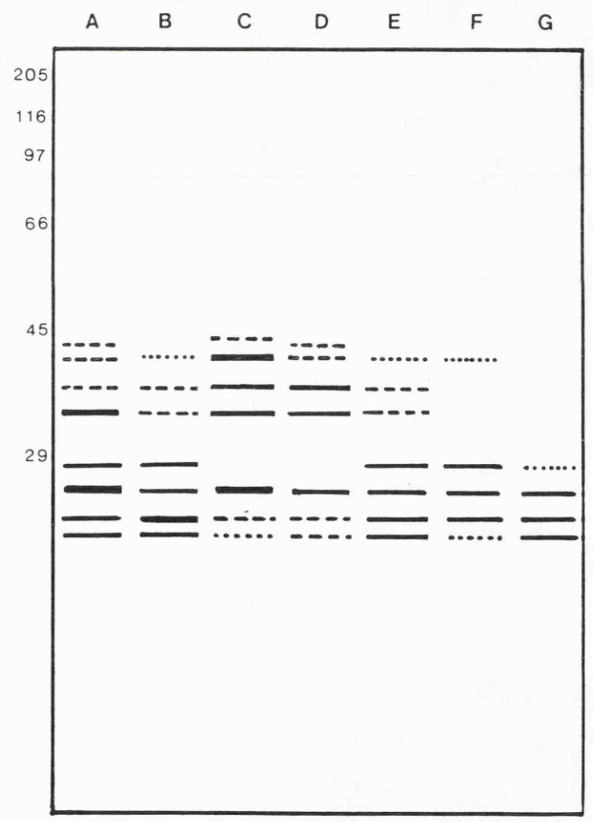
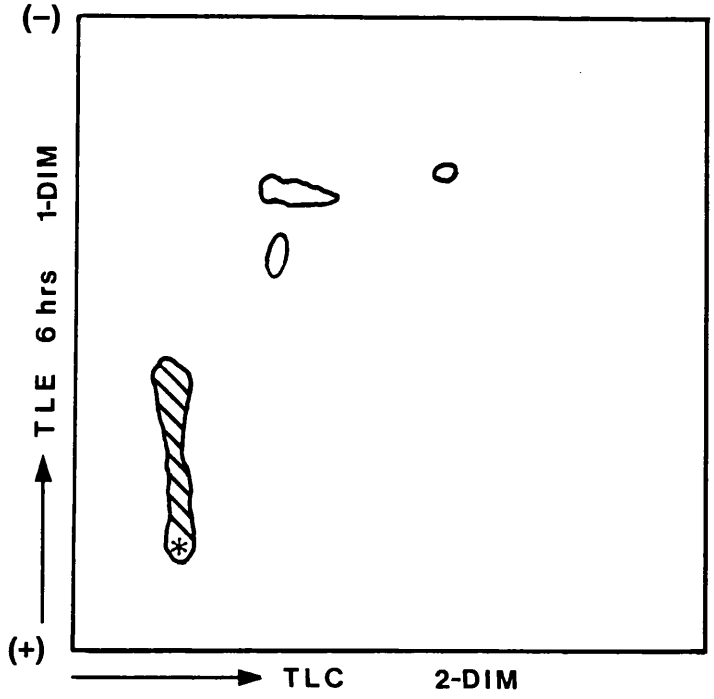
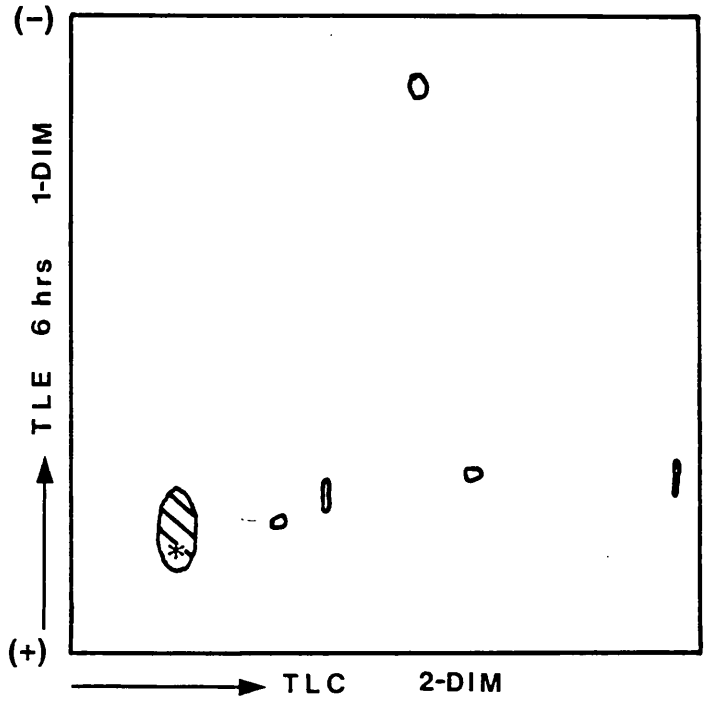


Figure 2.16. 2-D TLE/TLC peptide map of "control" proteins (a) galactosidase and (b) phosphorylase. Shaded areas show staining reaction after chlorination. For details of running conditions, see text (section 2.5.2).

a



b



method (figure 2.3.) This implies that the latter method results in greater cell degeneration. Furthermore tissue sections stained with haematoxylin and eosin (section 2.1.2.) showed poorer morphological preservation of tissues incubated by the submerged method (figure 2.4.) even with enhanced atmospheric conditions of 95% O_2, 5% CO_2. In addition, the increased cost and hazard of this technique using bottled oxygen, was a contributory factor against choosing this variation of the method.

2.6.2. Analysis of labelled glycoproteins.

Medium from both benign and malignant tissue incubated for 18 and 48 hours, when compared, showed a strikingly higher yield of radiolabelled products after 48 hours (figure 2.5). Coomassie blue stained gels also showed a greater overall yield of proteins after 48 hours although many proteins were clearly seen after only 18 hours (figure 2.6).

There were no significant differences in the viability of tissue from the two incubation time periods, and the viability of all tissues incubated for 48 hours was good, as assessed by morphology (section 2.1.).

Assessment of the incorporation of [^{3}H] glucosamine hydrochloride was made difficult by the severe blackening of the X-ray film above a molecular weight of 150 kD (figure 2.7). Reduction of the amount of label or exposure time resulted in a marked reduction in the detection of incorporated label into

the lower molecular weight glycoproteins.

Besides many similarities in the expression of [^{3}H] fucose and [^{3}H] galactose labelled glycoproteins released from benign and malignant breast tissue, major differences were also identified. A group of four glycoproteins in the molecular weight range 210-280 kD were identified in different combinations from all carcinomas whereas media from only 7 of the 13 benign samples contained glycoproteins within this range (table 2.2 and table 2.3). Densitometric profiles of selected cases quantitatively show these differences between galactose and fucose labelled glycoproteins from both benign and malignant breast tissue, (figures 2.8-2.11). Differences identified between glycoproteins below 200kD released from benign and malignant breast were less consistent; a glycoprotein of molecular weight 130kD was more prominent in carcinoma samples (figures 2.8 and 2.11) whereas a glycoprotein of 55kD was often more pronounced in benign samples (figure 2.10). A number of glycoproteins with molecular weights of 110, 96, 64, 47, 35 and 18kD, which labelled with [3H]fucose and [3H]galactose, were common to both benign and malignant specimens (figures 2.9-2.11).

In the benign cases glycoproteins below 200kD, (with the exception of the glycoprotein at 130kD), incorporated both [3H]fucose and [3H]galactose (figure 2.10). The expression of the high molecular weight (>200kD) glycoproteins in benign cases was small (7/13), but appeared to relate to the degree of

hyperplastic change (table 2.3). There was also a limited incorporation of [^{3}H]fucose (2/14) into the high molecular weight glycoproteins. Furthermore the glycoprotein of 230kD was notably absent from these glycoprotein profiles.

In the malignant cases there was a high degree of heterogeneity in the glycosylation of glycoproteins released from carcinomas, irrespective of molecular weight. There was a marked association between the expression of the high molecular weight glycoproteins and malignancy, particularly the glycoprotein of 230kD, with 17/27 malignant cases showing expression compared to 0/13 for benign cases. There was also an association between the fucosylation of these glycoproteins and malignancy; of those benign cases expressing the high molecular weight group of glycoproteins, only 2/7 showed incorporation of [^{3}H]fucose compared to 26/27 for the malignant cases. Although there did not appear to be a strong association between glycoprotein expression and differentiation in malignancy, the proportion of glycoproteins expressed was slightly higher in poorly differentiated tumours. Data for the separation of high molecular weight glycoproteins for the one case, a secretory carcinoma (no. 525) was not available. This was due to impaired migration of SDS solubilized material from the stacking gel, which was probably related to the complex mixture of glycoproteins that are characteristic of this rare type of tumour. Node sampling had not been performed for several of the

carcinomas, limiting any comparisons. However comparison of a lymph node metastasis with its primary carcinoma for one case showed that the pattern of expression for [^{3}H]fucose labelled glycoproteins was similar (figure 2.7b).

2.6.3. Protein blotting.

The results from lectin blotting showed similar patterns to the fluorographs. Blotting with wheat germ agglutinin (WGA) however, provided better resolution of the high molecular weight glycoproteins (figure 2.12, lanes A-C) and was therefore easier to interpret than the fluorographs showing [^{3}H]glucosamine incorporation. A number of high molecular weight glycoproteins, >205kD were visible in both benign and malignant cases, although there was significant heterogeneity between cases (lanes B and C). Peanut agglutinin (PNA) binding was greatly enhanced after treatment with neuraminidase to remove terminal sialic acid residues (figure 2.12, lanes D-F), thereby exposing the PNA binding domains. (Figure 2.13 shows the effect of neuraminidase pre-treatment on PNA binding). The major difference between benign and malignant cases was in the high molecular weight glycoproteins, (as seen from fluorography). In the poorly differentiated carcinoma (figure 2.12 lane D), there was strong binding of PNA to a glycoprotein of molecular weight 230-250kD. This was not seen in the benign case (lane E) and showed only faint reactivity to a glycoprotein of similar mobility in another

benign case (lane F). In addition there were a number of quantitative differences in the lower molecular weight glycoproteins (45-116kD) between the benign and malignant cases. Blotting with the Ulex europeaus agglutinin (UEA) also yielded similar patterns to fluorography, although binding in the high molecular weight regions was less apparent (lane G, arrows highlight faint bands at 230kD and 250kD). The binding patterns of lower molecular weight glycoproteins (<205kD) was similar between benign and malignant cases with some quantitative differences, despite equivalent protein loading, observed at 40kD, 43kD and 70-200kD (figure 2.12 lanes G and H).

Protein blotting with the monoclonal antibodies HMFG1 and HMFG2, showed significant binding of both antibodies to the malignant cases but not the benign cases (figure 2.14). The HMFG1 antibody reacted faintly with a high molecular weight component only in two of the three malignant cases, which appeared also to be reactive with HMFG2. This antibody bound two high molecular weight components, the lower of which was a similar molecular weight range to the group of glycoproteins previously seen by fluorography.

2.6.4. Peptide mapping.

Due to the small quantities of glycoprotein available the non-enzyme method was used (see section 2.5.1). The resultant heat cleaved peptide map showed after silver staining only very faint bands which, despite using a variety of techniques (Appendix D),

could not be reproduced in a satisfactory photograph format. The peptide map has therefore been presented in diagramatic form (figure 2.15). From this peptide map there appears to be considerable qualitative similarity between the [^{3}H]fucose and [^{3}H]galactose labelled glycoprotein of 230kD from the same carcinoma (lanes A and B) and also between different carcinomas (lanes F and G). Similar peptide fragments are also seen in the glycoproteins of 250kD (lane E) and 280kD (lanes C and D) in the lower molecular weight group of bands (<29kD). The glycoprotein of 280kD labelled with either [^{3}H]fucose (lane C) or [^{3}H]galactose (lane D) were almost identical, with the exception of a slight difference in mobility of the 43-45kD peptide.

Peptide mapping using the 2-D thin-layer approach, although showing differences between the two test proteins (figure 2.16a,b), did not show consistency between individual runs of the same test proteins. This technique was therefore abandoned as being unsuitable as a procedure for obtaining peptide mapping data in this study.

2.7. Discussion.

The work has shown that there are significant differences in the nature of glycoproteins released from benign and malignant human breast *in vitro*, the major changes occurring with malignancy being related to glycoprotein size and fucosylation.

Organ culture, in which the stromal-epithelial relationships of the tissue are maintained, is a valuable method for studying the synthetic capacity of human breast (Wellings and Jentoft, 1972). This approach has previously been used for studying glycoprotein synthesis in breast (Dermer and Tokes, 1978; Walker, *et al.*, 1986; Jones and Walker, 1987). Previous tissue autoradiographic studies have shown that benign and malignant breast epithelium retain their capacity to synthesize glycoproteins for periods from 24 to 72 hours (Tokes and Dermer, 1977; Walker, *et al.*, 1986). In the present study there was a greater yield of radiolabelled products from the 48 hour cultures than the 18 hour cultures, despite comparable protein yields, indicating that there is a lag phase between uptake of [^{3}H] sugars, incorporation and release of glycoproteins. Moreover the similarities between the general protein products released after 18 hour and 48 hour culture, suggests that any differences are not related to differences in cell membrane turnover. The radiolabelled products detected could be derived from the epithelium by two mechanisms; (i) active secretion by exocytosis, or (ii) shedding of membrane associated glycoproteins by membrane turnover or proteolytic cleavage.

The majority of the studies which have compared glycoprotein expression by benign and malignant breast tissue have utilized histological methods, e.g. the various immunohistochemical investigations using monoclonal antibodies which detect the components of

the MFGM (Arklie, *et al.*, 1981; Foster, et *al.*, 1982a and 1982b; Hilkens, *et al.*, 1984), and lectin histochemistry (Walker, 1984a, 1984c and 1984d; 1985). Whilst these approaches are of value for determining cellular heterogeneity they cannot identify the molecular heterogeneity which has been found by immunoblotting of electrophoretically separated breast cell and tumour homogenates (Burchell, *et al.*, 1983; Turnbull, *et al.*, 1986).

Increased expression of higher molecular weight glycoproteins occurring with malignancy has been reported for animal tumours (Smets, *et al.*, 1977), including differences between rat mammary adenocarcinomas of low and high metastatic potential (Steck and Nicolson, 1983), but comparable studies on primary human tissue have been limited. Previous studies examining the synthesis of [^{14}C]glucosamine-labelled glycoproteins by benign and malignant breast identified a group of acidic glycoproteins with molecular weights greater than 200kD which were more prominent in malignant specimens (Gendler, *et al.*, 1982). In the present study using metabolic labelling techniques with [^{3}H]glucosamine, the identification of these high molecular weight glycoproteins was not possible due to the severe blackening of the X-ray film. Similar problems have been experienced by other groups (Dermer and Tokes, 1978; Ng, *et al.*, 1987). It is likely that the use of tritiated glucosamine resulted in a higher incorporation of label into other hexosamines (e.g.

N-acetyl galactosamine, via the lactose biosynthetic pathway) and N-acetylneuraminic acid, thereby labelling, not one but several different constituents of the carbohydrate chain. Furthermore part of the blackening of the X-ray film in the high molecular weight ranges would be due to silver grain coalescence. In view of the difficulties in data interpretation of glucosamine labelling, a detailed approach using both lectins and antibodies to glucosamine linkages would provide more information. This was indeed the case when using WGA on Western blots.

In this study there was a lower incidence of glycoproteins in the region 210-280kD detected from benign than the malignant specimens. In the latter it was found that the number of glycoproteins identified within this range increased slightly with decreasing differentiation of the tumours, thereby supporting the relationship between increasing molecular weight size with malignancy (Jarnfelt, *et al.*, 1978; Richards, *et al.*, 1981; Smets and Van Beek, 1984). Differences in lower molecular weight glycoproteins released from benign and malignant breast were also identified. Both increased (e.g. 130kD) and decreased expression (e.g. 55kD) of glycoproteins was found from tumours, whilst a number of bands appeared to be consistent between all samples: The often distinct glycoprotein migrating with an apparent molecular weight of 64kD (figure 2.8) was assumed to be serum albumin which, despite tissue preincubations remained as a significant although

quantitatively variable band. Its presence served as an internal standard.

Variations in cellularity between benign and malignant samples did not appear to be a significant factor since the radiolabel incorporation into the lower molecular weight components released from both types of tissue, were similar.

Glycoproteins of differing molecular weights which are synthesized by mammary cells have been recognized by many workers. The band at 47kD could be the same glycoprotein previously identified with a molecular weight 48kD by Dermer and Tokes when using similar techniques (Tokes and Dermer, 1977; Dermer and Tokes, 1978). A common method of analysis has utilized monoclonal antibody technology by raising antibodies against individual components or whole cell homogenates from breast tumours, cell lines and MFGM's derived from human milk. Another glycoprotein identified in this study, having molecular weight 43kD, could be the same as that identified by Edwards, *et al.*, (1986) but only by virtue of its similar electrophoretic migration since it did not exhibit the tumour specificity suggested by their study. Of the higher molecular weight glycoproteins identified in this study, the one of 280kD may be the same or similar to that detected by Major, *et al.*, (1987) using antibodies derived from primary breast tumours and liver metastases. The similarity in the expression and electrophoretic mobility of the glycoprotein 230kD to the BCA-225 described by Mesa-Tejada, *et al.*,

(1988) was further investigated (see chapter 4) when antibodies became available commercially.

Besides the prominent differences in the frequency of high molecular weight glycoproteins (>200kD) released from benign and malignant breast, there were significant differences in relation to the incorporation of [^{3}H]fucose. The latter was a major feature of the high molecular weight glycoproteins released from carcinomas, although heterogeneity was prominent in some cases, but was essentially not observed in the glycoproteins released from benign breast in culture. Heterogeneity of fucosylation of lower molecular weight glycoproteins (40-80kD) was observed in both benign and malignant breast. Previous studies of blood group antigens (Kapadia, *et al.*, 1981; Walker and Day, 1986) using immunohistochemical techniques have shown differences in the expression of fucosylated glycoproteins between benign and malignant breast which may reflect quantitative and/or qualitative differences in specific fucosyl transferases or their regulation. Studies of some cell lines have noted changes in fucose metabolism which could have an influence on the biological behaviour of the cells (Steiner, *et al.*, 1983).

The glycoprotein of molecular weight 230kD was particularly notable for only being detected in carcinomas, where it was nearly always fucosylated. This was the only glycoprotein which showed such selectivity and has subsequently been the subject of more detailed studies in later chapters.

As mentioned earlier concerning the use of lectins in the study of glucosamine containing glycoproteins, binding of wheat germ agglutinin provided a better resolution of the high molecular weight regions than the [^{3}H]glucosamine metabolic labelling technique. A number of bands were readily discernable after lectin blotting and may be the same high molecular weight acidic glycoproteins previously described (Gendler, *et al.*, 1982). However unlike their findings, the glycoproteins appeared to be equally prominent in both malignant and some benign specimens. The remaining two lectins used generally paralled the findings of fluorography. Peanut agglutinin did not provide any significant additional information to the findings with [^{3}H]galactose. However since the lectin will interact with several different galactose disaccharides (Pereira, *et al.*, 1976) it cannot be assumed that glycoproteins of the same molecular weight from different tissues will possess the same glycan structure. A more detailed analysis of sugar content and glycosidase digestion would be required, in addition to the neuraminidase digestion that was found to be necessary.

The L-fucose binding UEA-I generally did not label the high molecular weight glycoproteins. This lectin has a very high affinity for H monofucosyl oligosaccharides of the type 2 chain (Debray, *et al.*, 1981), which may not have been present in the glycoproteins or they may have been di-fucosylated which would result in a reduction in binding affinity.

The spatial configuration of the oligosaccharide core also may make the fucosyl residues inaccessible to the lectin (Debray, *et al.*, 1981). Indeed this latter problem of steric hinderance is not limited to UEA-I binding alone, but is a general problem with all lectins, and therefore supports the use of the metabolic labelling approach.

Analysis by two-dimensional electrophoresis, glycosidase digestion (with lectin visualization), and peptide mapping is required to assess similarities/differences between the protein and glycan structures of glycoproteins isolated from different carcinomas. Moreover these approaches could be usefully extended to analyse the differences already observed in the glycoproteins released from benign and malignant samples. A variety of peptide mapping methods were tried in order to surmount the problem of limited amounts of tissue and hence purified glycoproteins. Since it had been reported that some proteins retained their antigenicity after fixing and staining, as determined after transfer to nitrocellulose (Phelps, 1984), attempts were made to produce peptide maps (where antigenicity was not a requirement) from glycoproteins previously subjected to fluorographic procedures. This was necessary as the glycoproteins of interest were only identified after fluorography. Although the method of Phelps (1984), or more rapid variations of it (Perides, *et al.*, 1985) were not tried, this may in retrospect have provided the sensitivity required beyond that achieved with the

silver stain. So as to increase the sensitivity of detection of peptide fragments, radio-labelling techniques were considered. Previous studies involving the radio-iodination of proteins both prior to, (Elder *et al.*, 1977), and after, elution (Bray and Brownlee, 1973) have shown that peptide mapping is possible after fixation and staining of proteins. However, due to the technical and safety considerations involved with radio-iodination procedures, labelling peptides with ^{35}S using a commercial sulpher labelling reagent (SLR) was considered. The reagent was subsequently found to be reactive with β-mercaptoethanol, water, SDS and cross-linked acrylamide, all of which were present, to varying degrees, in the gel slices containing the glycoproteins/peptide fragments to be labelled. The SLR reagent was subsequently used to label the polyclonal antisera (P5252), as described in chapter 4.

The main problem encountered with the peptide mapping in this study was the elution of peptide fragments from the gel slice, which in many instances was of high acrylamide concentration (10-20%). In order to maximize diffusion of the fragments from the gel slice an extended period of cleavage was tried, which would result in very small peptide fragments. It was anticipated that these peptide fragments would not be retarded in a high concentration acrylamide gel and therefore a combined thin layer electrophoresis/chromatographic analysis was tried. Solvent evaporation probably contributed to the

irreproducibility of this method and was subsequently abandoned in favour of the comparatively successful procedure described in section 2.5.1. The chemical and thermal insult that the "intact" glycoproteins were subjected to may have resulted in irreproducible and unreliable peptide maps. However, the successful use of dried and fluorographed gels has been described in a previous study, in which a different method of cleaving was used, (Saris, *et al.*, 1983). Indeed conformational changes in the glycoproteins resulting from the extreme conditions, may in themselves render the glycoproteins insensitive to other methods of cleavage (e.g. by enzymes), thereby making the heat-cleavage method (or any chemical cleavage method) the most reliable choice. This method resulted in similarities between the peptide maps of the three high molecular weight glycoproteins tested. The greatest similarities were observed between the peptide fragments detected below 29kD for the glycoproteins of 230 and 250kD. The similarities were consistent between different tumours and was independant of labelled sugar. The glycoprotein of 280kD showed only three of the four lower molecular weight bands (<29kD) common to the glycoproteins of 230 and 250kD, and differences in the bands detected between 30-45kD were predominantly quantitative. This preliminary mapping data indicates a marked sequence homology in the group of high molecular weight glycoproteins identified. The qualitative differences observed could be the result of changes in the protein

core or, glycosylation changes in the glycan chain. If the former is the case then greater differences in the peptide map would be expected, due to alterations in the position and number of cleavage sites present in the protein sequence. However if the differences were due to altered glycosylation of the glycoproteins this may still give rise to similar peptide maps, (assumming that the peptide cleavage conditions also caused cleavage of the glycan chains from the peptide fragments), and could explain the differences in molecular weights of the "intact" glycoproteins in SDS-PA gels. Moreover this would suggest that the association between these high molecular weight glycoproteins and malignancy, is a glycosylation dependent phenomenon. One of the major points of interest is the nature of the protein cores, since this would indicate whether the higher molecular weight glycoproteins released from carcinomas are formed completely *de novo* or whether they represent aberrant glycosylation of glycoproteins formed by normal breast. Data from the analysis of mucins expressed by benign and malignant breast cells (Burchell, *et al.*, 1987), indicates that the latter is more likely. Changes in the carbohydrate composition of glycoproteins involved in the function of normal cells could be of significance in determining the altered behaviour of malignant cells.

CHAPTER 3

ALTERED EXPRESSION IN-VITRO OF HUMAN BREAST TUMOUR ASSOCIATED GLYCOPROTEINS

This chapter is comprised of two parts both concerned with *in-vitro* modifications of the glycoproteins previously identified from cultured breast tissue, (see chapter 2). Part A covers the modulation of glycoprotein expression by two hormones and part B the effects of glycosylation inhibition by tunicamycin.

Part A.

There has been increasing interest in the concept of modulation of phenotypic expression of antigens in breast carcinomas (McGuire, *et al.*, 1985). The ability to, (i) stimulate differentiation in breast carcinomas, and hence potentially modify tumour behaviour in vivo, or (ii) to suppress tumour associated antigens that may be involved in aggressive tumour behaviour, would be of great clinical value. Many previous studies in this area have used

modulating agents such as interferon, sodium butyrate, and retinoic acids although these studies have been confined to general growth effects observed in culture (Lotan, 1979; Schroder, *et al.*, 1983; Marth, *et al.*, 1985). In the assessment of modulation different approaches have been used; immunohistochemical (Jones and Walker, 1987), biochemical (Grieve, *et al.*, 1980; Abe and Kufe, 1984) and fluorescence activated cell sorting (FACS) analysis (Greiner, *et al.*, 1984; Marth, *et al.*, 1985). Most of the studies of modulation in human breast cancer have been confined to cell lines (Marth, *et al.*, 1985; 1987; Daxenbichler, *et al.*, 1986; Wetherall and Taylor, 1986), with only a few studies using primary tumour tissue (Jones and Walker, 1987). The latter study observed the effects of the peptide hormone insulin and the corticosteroid hormone hydrocortisone on breast carcinomas in organ culture. They showed modulation of the MFGM, in a small proportion of cases, using the two antibodies HMFG1 and HMFG2, which react with epitopes on the MFGM. The study, like that by Turnbull, *et al.*, (1986), showed that the expression of both the HMFG1 and HMFG2 determinants are relatively constant in most tumours but variable and subject to modulation in others.

Using these hormones, it was decided to assess what modulating effects could be observed, using a biochemical approach, on the expression of those glycoproteins previously identified in chapter 2. It was expected that the type and extent of any modulation of expression could confirm the

relationship of these glycoproteins as tumour associated antigens. To determine whether the primary hormonal effects of hydrocortisone or insulin were due to cell proliferation the uptake of bromodeoxy uridine was measured.

The work in this section was the subject of a poster and an oral presentation given to the 627th Biochemical Society meeting (Rye & Walker, 1988) and part of an oral presentation to the 158th meeting of the Pathological Society of Great Britain and Ireland, (Appendix G).

Part B.

Analysis of glycoproteins using glycosylation inhibitors is a well established technique in determining many factors, such as the structure and function of oligosaccharides in glycoproteins. Many such inhibitors have been identified for use in the assessment of N-glycan chain synthesis and processing; tunicamycin (Takatsuki, *et al.*, 1975; Tkacz and Lampen, 1975), deoxynojirimycin (Saunier, *et al.*, 1982), nojirimycin (Hettkamp, *et al.*, 1982), castanospermine (Saul, *et al.*, 1983), and swainsonine (Dorling, *et al.*, 1980; Arumugham, *et al.*, 1983). Excepting tunicamycin, these inhibitors act on the protein-bound precursor oligosaccharide by preventing the action of the glucosidases or mannosidases ("trimming" enzymes). Tunicamycin, an antibiotic isolated from *Streptomycin lisosuperificus* (Takatsuki, *et al.*, 1971), inhibits the first step in the N-glycan

biosynthetic pathway, by preventing the formation of the lipid-linked oligosaccharide precursor, (Takatsuki, *et al.*, 1975; Tkacz and Lampen, 1975). Although other antibiotics have been reported to inhibit the same reaction they are less specific (Takatsuki and Tamura, 1982). This inhibitor has been used extensively to study the importance of glycosylation in a number of cell types (Olden, *et al.*, 1978; Damsky, *et al.*, 1979; Gibson, *et al.*, 1978; Parent, *et al.*, 1982).

In this study the effects of tunicamycin on glycoprotein expression from human breast in organ culture, was examined as described in section 3.2. The tissues from organ culture were processed for autoradiography to check the distribution of altered expression, throughout the tissue blocks, resulting from tunicamycin exposure.

(Part A) 3.1. Glycoprotein Modulation by Hormone Additives.

(Part B) 3.2. Glycoprotein Modification by Tunicamycin.

3.3. Results.

3.4. Discussion.

3.1. Glycoprotein Modulation by Hormone Additives.

3.1.1. Organ culture.

Eight primary breast carcinomas were cultured using the raft organ culture technique as described in chapter 2, section 2.2.1. All tissues were incubated with [^{3}H]fucose (20uCi/ml) with either no hormone additives (control), insulin (5.0ug/ml) or hydrocortisone (1ug/ml). Three hours before the end of the 48hr incubation period 10^{-2}M bromodeoxyuridine (BrdU) was added to each culture. After incubation the tissue and medium was processed as described in chapter 2, sections 2.1.2 and 2.3.1 respectively.

3.1.2. SDS-PAGE.

The procedures used were identical to those already described in the previous chapter, section 2.3.

3.1.3. BrdU immunohistochemistry.

Incorporation of bromodeoxyuridine was detected by an immunohistochemical method, utilizing the Avidin-Biotin Complex technique, (Hsu, *et al.*, 1981). This method amplifies the final signal thereby increasing the sensitivity which is particularly useful in detecting the incorporation of BrdU into mitotic cells. This particular technique was carried out by Mr. M. Rae in the department of Pathology.

Tissue from organ culture (section 3.1.1.) was processed (section 2.1.2.) and 4um sections were dewaxed, rehydrated and endogenous peroxidase activity blocked by incubating sections for 30 minutes in a 0.3% H_2O_2 solution in methanol. In order to increase

accessibility and prevent steric hinderance of the antibody by cross-linked nuclear proteins the tissue sections were incubated in 2M HCl for 30 minutes at 37°C. After incubation the acid was neutralized in 0.1M sodium tetraborate (pH 8.5) for 10 minutes then washed in PBS for a further 10 minutes before tissue sections were blocked with normal rabbit serum (1/5 dilution in PBS) for 5 minutes. The murine anti-BrdU primary antibody (1/10 dilution in PBS) for 1 hour at 37°C. After washing first in PBS then in TBS (Tris buffered saline) for 5 minutes each the secondary antibody, biotinylated rabbit anti-mouse (1/400 dilution in TBS) was applied. After 30 minutes incubation at room temperature, unbound secondary antibody was removed by rinsing in TBS for 5 minutes. The tertiary reagent comprising a mixture of complexed biotinylated peroxidase and avidin was applied for 30 minutes, rinsed with TBS and finally stained and mounted as described in section 4.3.

3.2. Glycoprotein Modification by Tunicamycin.

3.2.1. Organ culture.

The breast tissue was received, diced and pre-incubated as described in chapter 2, sections 2.1 and 2.2. For the two carcinoma cases studied in this way, 20 cultures for each radiolabelled sugar were prepared; 4 dishes for each of 5 time intervals over a 48hr period. After the 2hr pre-incubation, culture

medium in all dishes was replaced with fresh pre-warmed DMEM with penicillin (100U/ml) and streptomycin (100ug/ml). To 10 of the cultures 1ug/ml of tunicamycin was added. The remaining 10 cultures (controls) were not treated with tunicamycin but were otherwise identical to all other samples. After a 6hr incubation period the culture medium in all dishes was again replaced with fresh pre-warmed DMEM containing antibiotics, and 30uCi/ml of either [^{3}H] fucose or [^{3}H] galactose was added to all dishes. Organ cultures were then incubated in an atmosphere of 95% air 5% CO_2 at 37°C. Duplicate test (tunicamycin treated) and control (no tunicamycin) cultures were removed at 6,12,24,36 and 48 hours. For each time interval the tissue was fixed in Carnoy's fluid (6:3:1, ethanol: chloroform: glacial acetic acid) for 4hrs and processed as described in chapter 2 section 2.1.2. The medium from each time interval was collected and 1ul/ml of the protease inhibitor (PMSF) was added to give a final concentration of 1mMol/l. Samples were stored at -20°C until required, and subsequently dialysed and concentrated as described in chapter 2, section 2.3.1.

The standard formaldehyde based fixative was only used for one of the control sets of tissue to determine viability. Tissues for autoradiography were not fixed in this fixative as it has been shown to lower the sensitivity of the emulsion used in autoradiography (Flitney, 1977). An alcohol based fixative allowed subsequent autoradiographic

techniques to be performed without loss of sensitivity.

The N-linked glycosylation inhibitor, tunicamycin, has been used for periods up to 24hrs (Damsky, *et al.*, 1979; Savage & Baur, 1983), although it has been reported that high concentrations or long exposure to this drug can result in up to 80% inhibition of protein synthesis. Therefore only a 6hr pulse of tunicamycin at 1ug/ml was used. Radiolabel was added after tunicamycin so that any alterations in the expression of radiolabelled glycoproteins would be attributable to the effects of the tunicamycin pulse.

3.2.2. SDS-PAGE and fluorography.

The procedures used were identical to those already described in the previous chapter, section 2.3.

3.2.3. Autoradiography.

The glass microscope slides used in autoradiography are specially treated to ensure the optimum adhesion of the photographic emulsion to the slide. The slides were first cleaned by soaking in chromic acid overnight, followed by thorough washing in tap water and two 30 minute rinses in distilled water. The cleaned slides are dipped into a freshly prepared 0.5% w/v gelatin solution containing 0.05% w/v chrome alum ($K_2SO_4.Cr(SO_4)_3.24H_2O$) and dried in a dust free atmosphere. These gelatinized or "subbed" slides were stored at 4°C until required and used for

all the autoradiography procedures described.

Tissue sections from paraffin embedded blocks were cut at 4um and mounted on subbed slides. Sections were dewaxed in xylol (5 min) and hydrated to distilled water through 99%, 99%, 95% alcohols (5 min. each). The following procedures were done under a safelight (Kodak 1A filter). The nuclear emulsion K2 (Ilford) was melted at 43°C and added to an equal volume of a pre-warmed 0.02% v/v glycerol solution. The diluted emulsion was stirred slowly and left to stand to allow air bubbles to disperse (this was tested by dipping a clean slide and examining for bubbles). The slides were individually dipped into the emulsion and withdrawn slowly and steadily to ensure an even coating. The dipped slides were then allowed to dry on a level plate over ice for 10 min. then further incubated for 1hr at room temperature. The coated slides were placed in a light tight box containing fresh silica gel, left overnight at room temperature and then exposed at 4°C for 4 weeks.

After exposing, the slides were placed in diluted (1/5) Phenisol developer (Ilford), for 10 min. at room temperature. The slides were rinsed gently in distilled water for 60 secs. then fixed for 20 min. in two changes of 30% sodium thiosulphate ("hypo"). Slides were washed in running tap water for a further 20 min. and finally counterstained briefly (15 secs.) in Harris's Haematoxylin. Before mounting the slides using XAM, the sections were dehydrated through 95%, 99%, 99% alcohol and cleared in xylol (5 min. each).

3.3. Results.

3.3.1. Glycoprotein modulation by hormone additives.

Densitometry of the fluorographs for five of the eight carcinomas studied showed that the presence of insulin in organ culture led to a specific decrease in the detection of the high molecular weight glycoproteins, most notably the glycoprotein at 230 kD. Parallel incubations with hydrocortisone showed a marked increase of the glycoproteins at 230 and 250 kD for the same carcinomas one of which also showed an increased expression of a glycoprotein at 110 kD when compared with the control tissues without hormones (figure 3.1). The responsiveness of the carcinomas appeared to relate to their histological differentiation; the five carcinomas showing modulation were all moderate to poorly differentiated tumours (grade II to III). Of the three carcinomas tested that showed no response to the hormone incubation, two were well differentiated (grade I) and one was moderate (grade II).

Examination of haematoxylin and eosin stained tissue sections showed that tissue viability was good with no major areas of necrosis.

The rates of proliferation, as determined by the uptake of BrdU in culture, were elevated with hydrocortisone in two of the three cases initially tested (table 3.1.). Only one of these cases showed modulation with the hormones. Insulin did not appear to have any effect on proliferation.

Figure 3.1. Densitometry traces of [3H]fucose-labelled glycoproteins released from organ culture of primary breast carcinoma; after incubation without hormones (a); with insulin (5.0ug/ml) (b); with hydrocortisone (1.0ug/ml) (c). Arrowed peaks, from left refer to the glycoproteins of 250, 230 and 110kD.

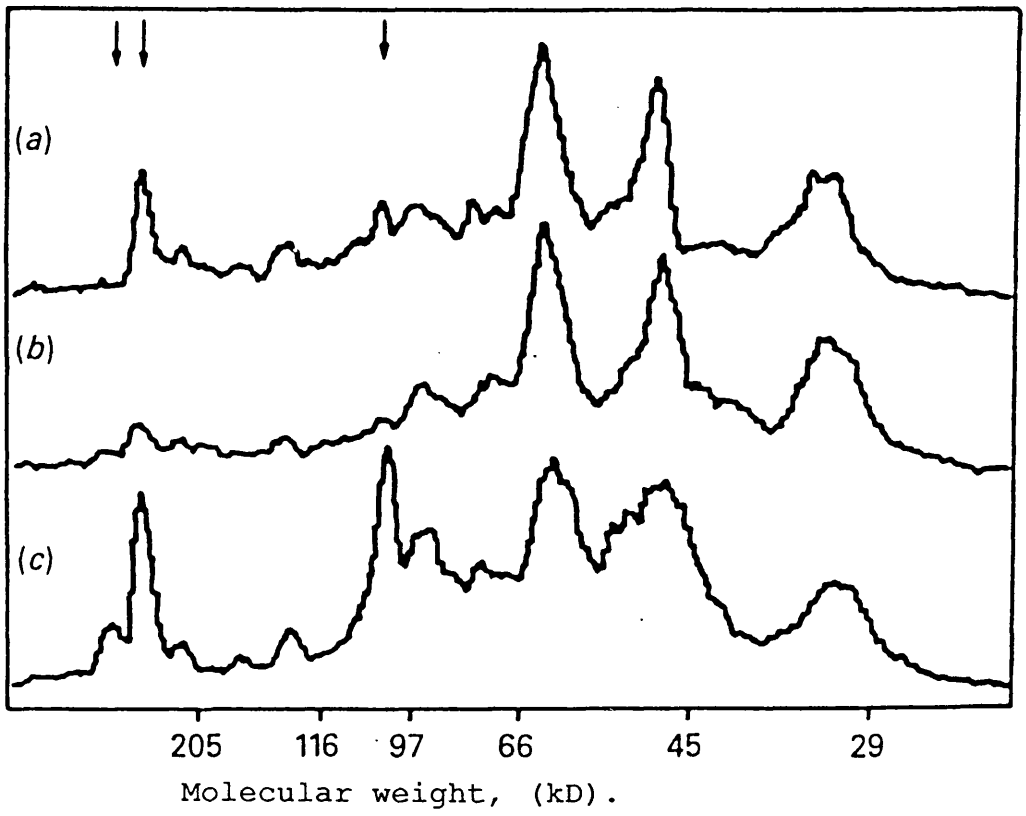
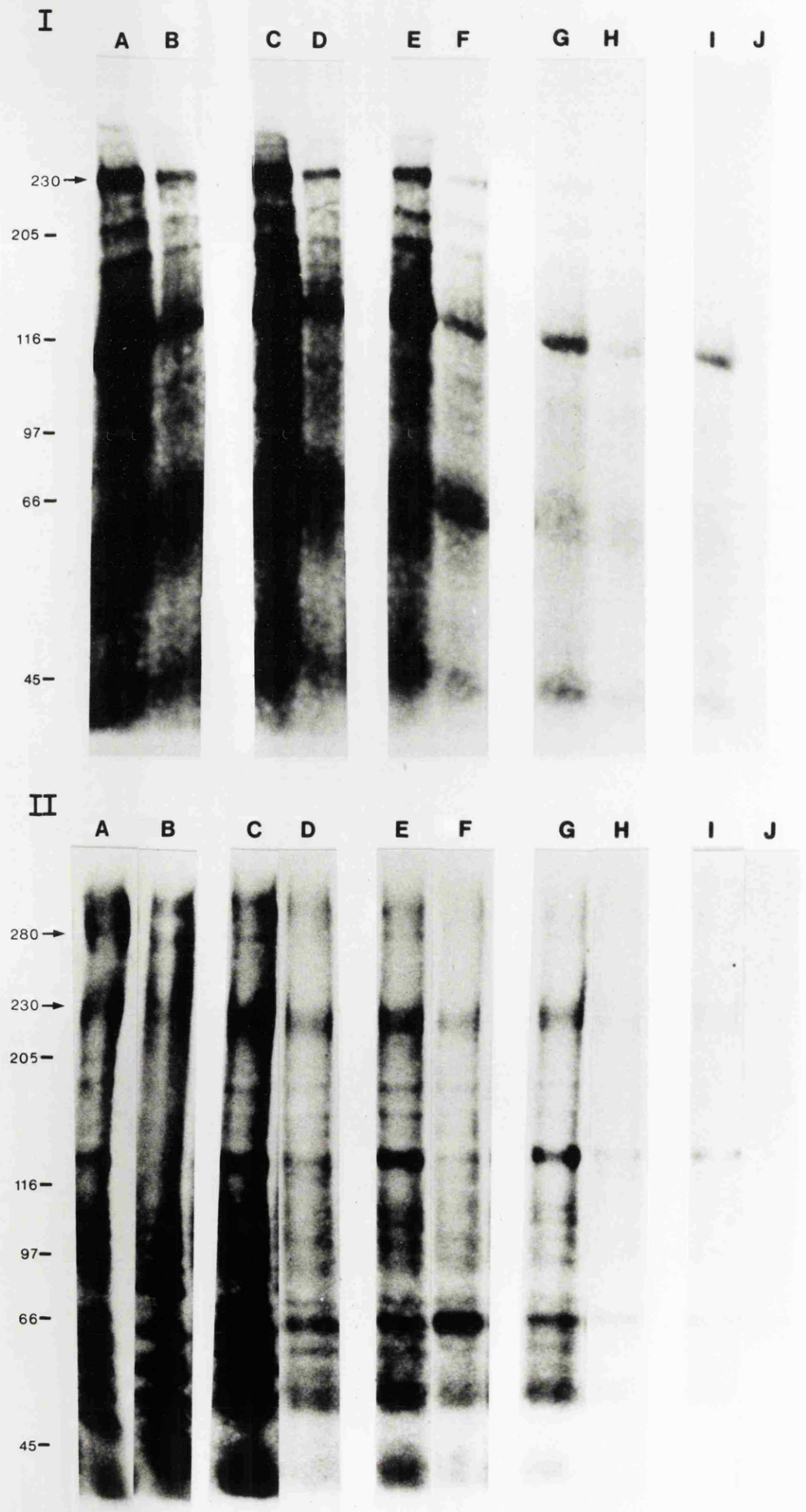


Table 3.1. Cell counting data from BrdU incorporation studies. Cell counts were determined from ten randomly selected fields per tissue section.

Case No.	BrdU labelling per total No. epithelial cells	Hormone conditions	Mitotic index (Im)
666	7/595	none	0.0117
	7/412	insulin	0.0169
	4/391	hydrocortisone	0.0102
673	3/840	none	0.0036
	4/433	insulin	0.0092
	22/779	hydrocortisone	0.0282
675	2/653	none	0.0031
	4/538	insulin	0.0074
	14/597	hydrocortisone	0.0234

Figure 3.2. Fluorograms showing the effects of tunicamycin on the release of [3H]fucose (I) and [3H]galactose (II) labelled glycoproteins from organ culture of primary breast carcinoma for 48hrs (A and B), 36hrs (C and D), 24hrs (E and F), 12hrs (G and H) and 6hr (I and J) incubations; with tunicamycin (B,D,F,H and J), without tunicamycin (A,C,E,G and I).



3.3.2. Glycoprotein modification by tunicamycin.

The expression of fucose and galactose labelled glycoproteins were significantly altered with the presence of tunicamycin in the culture medium (figure 3.2). This effect was particularly marked at the lower time interval (t=6), but also still visible after 36 hours culture. The overall effect seen with the tunicamycin pulse for both fucose and galactose labelled glycoproteins was a quantitative reduction in expression for all glycoproteins. This was particularly notable in the prominent fucose labelled glycoprotein at 230/250kD which was markedly reduced in intensity relative to the control samples even after 48 hours culture (figure 3.2a. lane B). The galactose labelled glycoproteins were significantly more affected by the tunicamycin, with many bands not appearing until 24-36 hours culture (figure 3.2b. lanes D and F). In the galactose labelled glycoprotein profile only, the 48 hour tunicamycin treated sample (figure 3.2b, lane B), shows a number of differences when compared to the 48 hour control (lane A) and to the earlier tunicamycin treated sample at the time interval of 36 hours (lane D). These particular differences are not apparent in the fucose labelled, treated and untreated samples.

3.3.3. Autoradiography.

Despite the changes described in section 3.3.2. the silver grains observed in the tissue autoradiography sections showed an even distribution

throughout the individual blocks of tissue for all incubation times. A general increase in silver grain numbers was observed with increased time both for the control and tunicamycin treated tissue.

3.4. Discussion.

Part A.

Previous studies, (Jones and Walker, 1987), have shown a better functional differentiation in certain carcinomas after exposure to insulin. These studies showed an enhanced expression of differentiation related markers and suppression of tumour-associated antigens. Based on these findings the specific modulation of the high molecular weight glycoproteins identified in chapter 2 support the earlier thesis that these glycoproteins are tumour associated antigens and not differentiation antigens. This is evidenced by the marked suppression of the glycoprotein of 230kD by the hormone insulin. The effects of insulin on the growth of cells in culture has been the subject of many early studies (Gey and Thahimer, 1924; Lasfargues, 1962). In both animal and human studies insulin has been reported to stimulate the growth of breast cancer cells (Kim and Furth, 1976; Welsch, *et al.*, 1976). Insulin receptors can be detected in 90% of carcinomas (Holdaway and Friesen, 1977), but the regulation of receptor concentrations has been found to be abnormal in several cell lines

studied (Mountjoy, *et al.*, 1983) and this alters the sensitivity of the cells to hormonal stimulation.

Modifications of the high molecular weight glycoproteins identified in chapter 2 have been demonstrated, in particular the glycoprotein of 230kD which has shown significant alteration in expression in the presence of insulin. The suppression of this glycoprotein could be due to the general differentiating effects of insulin described by Lasfargues (1975), or more specific effects relating to the biosynthesis of the glycoproteins.

Glucocorticoid hormones are found in a variety of normal tissues and cells; colon, kidney, leucocytes, liver, lung, placenta and prostate (Rousseau, 1984). The phenotypic response however, differs between tissues. Some tissue glucocorticoids induce *de novo* synthesis but in others a catabolic effect is seen. In many tumour cell systems corticoid hormones have been found to exert an anti-proliferative effect, although there is much controversy in this area with many studies claiming no effect in breast carcinomas (Vorherr, 1980). Indeed the controversy itself may be due to poor patient sampling methods: The plasma and urinary levels of glucocorticoids fluctuate with time of day, body weight, food intake, physical and emotional stress, pancreatic and hepatic function. Furthermore the presence or absence of receptors to these hormones in individual tumours further complicates this issue. Since these factors have not been accounted for in

many of the studies on breast cancer patients, the data must be viewed with sceptism. These factors in combination with the histological variations between tumours, could explain the variable responsiveness in this study of some carcinomas to the hormones. In the carcinomas where an effect was seen with hydrocortisone, only the glycoproteins of 230 and 250kD, and also one at 110kD, were elevated in their expression when compared with the control incubations. The elevated expression of the glycoprotein of 110kD was not found in any other samples tested and was not apparent in the glycoprotein profiles from the earlier study described in chapter 2.

These preliminary findings support the hypothesis from chapter 2, that the fucosylated glycoproteins in this high molecular weight group, in particular the glycoprotein of 230kD, are tumour associated.

Part B.

The specific effects on glycoprotein expression from carcinomas incubated in the presence of tunicamycin indicate that the high molecular weight glycoproteins identified in chapter 2 contain significant numbers of asparagine linked oligosaccharide chains containing fucose.

The distribution of silver grains in the cultured tissue blocks, as detected by autoradiography, indicated that the tunicamycin effects were even throughout the tissue.

It was unexpected to see a decrease in the majority of glycoproteins expressed after treatment with tunicamycin, indicating that any O-linked glycoproteins were either not separated in this system or they were equally affected by the tunicamycin's depressive effect on protein synthesis. The former could be due either to; (a), the O-linked mucins or glycoproteins not migrating through the resolving gel (despite only being 7.5%); (b), they were not expressed in large amounts into the culture medium; (c), or that they did not readily incorporate the labelled sugars. It is unlikely that the tunicamycin had any significant effect on protein synthesis, since the appearance of radiolabelled products after 12 and 24 hours was compatible with earlier studies in chapter 2, when radiolabel uptake and expression in glycoproteins released from 18 and 48 hour organ culture is compared. Furthermore the concentration and exposure of the antibiotic was similar to levels reported in other studies (Olden, *et al.*, 1978; Damsky, *et al.*, 1979). However, a control experiment observing the incorporation of [^{3}H]leucine, with and without tunicamycin treatment, would have been a more reliable indication of potential tunicamycin effects on protein synthesis.

The altered glycoprotein profile seen in the galactose labelled tunicamycin treated sample at 48 hours shows a number of glycoproteins in the molecular weight range 40-80kD, which were not seen in the other controls or earlier times. This is probably due to

increased proteolysis. The increased degradation may be due to an altered protein conformation resulting from the lack of carbohydrate, or to a decreased protection from proteases by beta-turns in the polypeptide, which are thought to occur at glycosylation sites (Beeley, 1977). The increased rate of degradation in glycan defective proteins is also well documented (Goldberg, *et al.*, 1975; Weitzman & Scharff, 1976; Olden, *et al.*, 1978). Moreover the reduced expression of specific glycoproteins, after treatment with tunicamycin, can be directly correlated to an enhanced proteolytic degradation (Yamada & Olden, 1982; Olden, *et al.*, 1982). The increased proteolysis in this one sample may be related to the serum-free conditions of culture, which have been associated with cell death and consequent release of vast quantities of enzymes (Leibovitz, 1986). However, these effects were thought not to be significant in this study since, (i) only one sample was affected, but all were in serum free conditions, and (ii) residual serum factors present in the tissue blocks would in part compensate for the absence of the serum supplement in the culture medium.

Future studies in this area of work would utilize the specific glucosidase and mannosidase inhibitors, castanospermine and swainsonine respectively, to study the structure-function relationship of these high molecular weight glycoproteins. These inhibitors are apparently less toxic than tunicamycin in tissue culture systems

(Fuhrmann, et al., 1985), and could avoid some of the problems associated with protein synthesis as described above.

CHAPTER 4

POLYCLONAL ANTIBODY PRODUCTION AND IMMUNOHISTOCHEMISTRY.

As a further step in the characterization of the glycoprotein of 230kD a polyclonal antiserum (P5252) was raised. Polyclonal antisera can be polyspecific or monospecific depending on the antigen preparation used for immunization. Polyspecific or multispecific antisera may be raised against a complex mixture of cellular components, e.g., membrane fraction (Bjerrum and Bog-Hansen, 1976), for the purpose of observing general changes in antigen population. For many immunohistochemical studies it is more advantageous to produce a monospecific antiserum (Walker, *et al.*, 1976) than employing hybridoma technology to produce a monoclonal antibody (Kohler and Milstein, 1975; Kennett, 1980). In the latter case a given clone of monoclonal antibodies will bind only with a specific epitope on an antigen, however a monospecific antisera contains a number of antibodies

that bind to a variety of epitopes on a single antigen. The advantage of the latter is clear when trying to identify the expression of a complex antigen such as a high molecular weight glycoprotein. Furthermore this type of antiserum is more likely to detect the glycoprotein molecule despite the micro-heterogeneity often seen in the glycan chains of glycoproteins expressed from tumours.

The polyclonal antiserum raised was used to examine the expression of the glycoprotein of 230kD in a wider range of breast tumours and also tissue from other sites using immunohistochemical methods. This immunohistological approach enabled a large number of tissues to be screened quickly. The indirect method (Coons, *et al.*, 1955), using the enzyme peroxidase as a label, is a simple two stage antibody technique. The method employs a primary unlabelled antibody (or antisera) which is applied to the tissue section first, and the excess is washed off. A second, labelled, antibody from another species, raised to the IgG of the animal donating the primary antibody, is then applied. This method is more sensitive than a direct approach and does not therefore require every individual primary antibody to be labelled.

The antisera raised against the glycoprotein of 230kD was also used in Western blotting methods to determine the molecular weight of the components reacting with the antisera. A number of antibodies known to react with antigens of molecular weights similar to the glycoprotein of 230kD were used in

order to compare their reactivity with that of the polyclonal antiserum P5252. These antibodies included; the HMFG2 antibody, reactive against a group of high molecular weight mucin-like components released from breast epithelia (see chapter 1, section 1.4.2.); fibronectin antiserum, binding a single glycoprotein of molecular weight 220kD; and BRST-1, also known as CU18 (Mesa-Tejada, *et al.*, 1988), a monoclonal antibody reacting with the glycoprotein BCA-225 (of apparent molecular weight 225-250kD) expressed in culture medium from the breast carcinoma cell line T47D.

Because of the binding of these antibodies to components of similar molecular weights to that found with P5252, as detected by Western blotting, competition studies were performed. An immunohistological approach was used in order to assess the specificity of the polyclonal antiserum in relation to the monoclonal antibodies HMFG2 and BRST-1.

4.1. Purification of Glycoprotein GP230/250.

4.1.1. Sample Preparation and Purification.

Separation was performed using the SDS-discontinuous gel electrophoresis system described in section 2.3.3 with the exception that a 1.5mm thick gel was used and the stacking gel was cast using a 1.5mm thick Teflon comb, designed by the author and made by the University workshop (Appendix A).

Medium derived from twelve cultured breast carcinomas known to contain the glycoprotein of 230kD (as determined by those studies described in chapter 2) were pooled. Equal volumes of sample and SDS-sample buffer (section 2.3.3) were heated together at 100°C for 3 minutes. The sample mixture was allowed to cool, centrifuged at 6000xg for 5 minutes and then applied to the trough in the stacking gel. High molecular weight standards were added to one of the *check* wells and 100ug total protein of sample to the other. The gel was electrophoresed as described in section 2.3.5. After electrophoresis the two *check* lanes were excised from the gel, stained (section 2.3.7) and prepared for fluorography (section 2.3.9). The remainder of the gel was wrapped in foil and stored at -20°C until the fluorographic record was obtained.

Using the fluorograph and molecular weight estimations (corrected for minor alterations in gel size found between the fixed CB stained gel and the previously frozen gel), the strip containing the glycoprotein of 230kD was cut from the thawed gel and subsequently used in the immunization protocol (section 4.2.3.).

4.2. Polyclonal Antibody Production.

4.2.1. Animals.

Three male New Zealand White rabbits were selected, each weighing about 2.5kg. Prior to inoculation the animals were allowed a 2 week "settling in" period. The weight and core temperature of each animal was recorded daily for three days immediately before starting the immunization protocol, and at least twice every week thereafter. After inocculation of antigen each rabbit was monitored closely for 4 days. Throughout the experiment all animals were regularly observed and checked for lameness, imobility or lumps at the injection sites.

4.2.2. Pre-screening animal sera.

Serum samples from each animal were taken after the settling in period but before immunization. A fluorescent antibody technique was utilized to screen the serum for any pre-existing activity in gut and breast tissue. Cryostat cut sections were dried overnight in the presence of silica gel at 4°C fixed for 5 minutes in acetone and washed in Tris buffered saline (TBS) containing 0.1% BSA. Tissue sections were blocked for 30 minutes with normal swine serum (NSS), 1/5 dilution. Tissue sections were incubated with serum from each animal for 2 hours at room temperature at the following dilutions; 1/40, 1/20, 1/10 and neat serum. After incubation the sections were washed twice in TBS/BSA for a total of 30 minutes. The secondary

antibody, swine anti-rabbit immunoglobulin antiserum conjugated with fluorescein isothiocyanate (FITC) was incubated at 1/100 dilution for 30 minutes at room temperature, washed as before and finally mounted in 25% 1,4 diazabicyclo[2.2.2]octane (DABCO), 90% glycerol/PBS. To prevent the mountant from drying out the edges of the coverslips were sealed with nail varnish. Positive controls included fibronectin and secretory piece polyclonal antibodies. Sections were observed immediately and photographed, when appropriate, on Ektachrome 400 using dark field illumination.

4.2.3. Immunization Protocol.

All invasive techniques involving the animals were carried out by Mr. P. Husken under the author's supervision. The work was covered by the Home office licence, No. ED 1110.

For bleeding, the animals were wrapped securely in an old green laboratory overall, taking care to keep the head free and preventing backward movement from the hind quarters. This method of restraint was thought to cause the least stress to the animal. A pre-immune bleed of 5ml was taken from each animal. The site of this pre-immune bleed and subsequent bleeds was the marginal vein situated at the dorsal edge of the ear. The hair at the edge of the ear was shaved using a razor blade, and sterilized using Pevidine. Alcohol is commonly used in many protocols but it tends to sting when the vein is cut and

Figure 4.1. a. Double needle apparatus for adjuvant/antigen mixing.

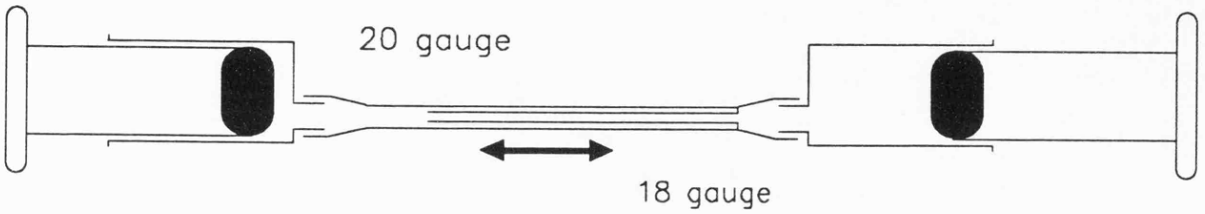
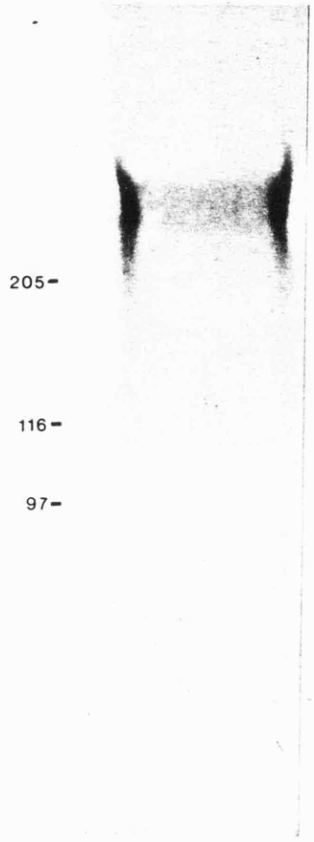


Figure 4.1. b. Glycoprotein preparation used for injection.



consequently is uncomfortable for the animal. To enhance the dilation of the marginal vein (not always necessary), a small swab i.e. a Q-tip or cotton bud, of xylene was applied to the tip of the ear. Holding the rabbits ear horizontally a small cut was made across the vein using a no. 5 scalpel blade. Blood was collected in a glass vial. After bleeding a cotton wool swab was applied with pressure to the cut vein for about 30 seconds. The xylene, if used, was washed off with hibitone. The animal was placed back in its cage and checked 15 minutes later to ensure bleeding had not restarted.

The antigen was prepared as described in section 4.1.1. The polyacrylamide gel slice containing approximately 300ug of purified glycoprotein was minced finely in 0.5ml saline. After adding 1.5ml complete Freunds adjuvant (FCA) the mixture was further emulsified by aspirating back and forth through a 20/18 gauge double needle (figure 4.1). This antigen mixture was administered by intramuscular injection, 0.25ml into each hind leg of all three animals. At 14 and 32 days after immunization a booster injection was given using the procedure described earlier for the primary inocculation, with the exception that incomplete Freunds adjuvant was used. A 5ml test test bleed was also taken from each animal.

4.2.4. Total serum Ig determination.

The single radial immunodiffusion (SIRD)

technique of Mancini was used (Hudson & Hay, 1980), and performed in duplicate for each animal. Glass microscope slides (76x53x1-1.2mm, BDH) were pre-coated with a 0.5% agar solution. These coated slides were allowed to dry and were then stored at room temperature until required. The pre-coating of slides in this way was necessary to hold the agar gel in place during the washing and staining steps.

A 2% agar solution using the barbitone buffer, prepared as described in Appendix B, was made by dissolving the agar in the hot buffer. Agar of electrophoretic purity was used since bacteriological agar was known to contain many impurities likely to interfere with the results. The barbitone agar was melted and 1ml aliquots transfered to six pre-warmed test tubes at 56°C. The working dilution of the goat anti-rabbit Ig antibody required to produce the optimum sized precipitin rings was determined empirically. An antibody dilution of 1 in 30 was found to be optimum. The antibody was diluted in PBS, warmed to 56°C and added to the agar aliquots at a ratio of 2:1. This solution was thoroughly mixed and the aliquots poured onto six pre-coated glass microscope slides and allowed to set for about 30 minutes on a level surface. Ten equally spaced 3mm diameter wells were cut in the gel, taking care to ensure the sides of the wells were vertical. The agar plugs were removed with a pasteur pipette attached to a vacuum line. Calibration standards containing rabbit Ig at concentrations 50, 100, 150, and 200ug/ml were added

Figure 4.2a. Example of Mancini plate for rabbit 5252-3.

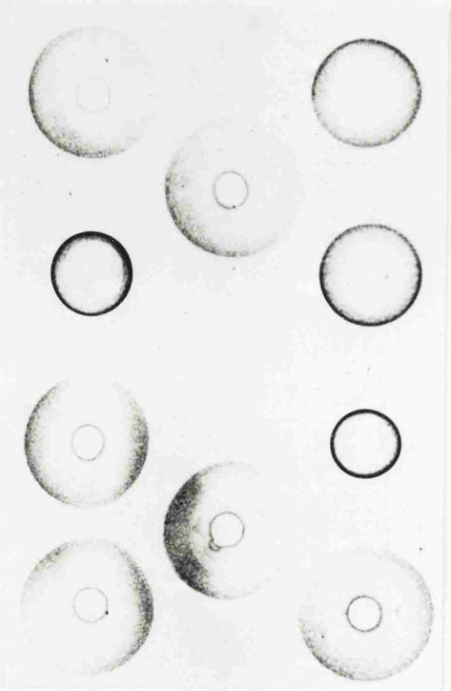


Figure 4.2b. Calibration curve derived from the Mancini plate above.

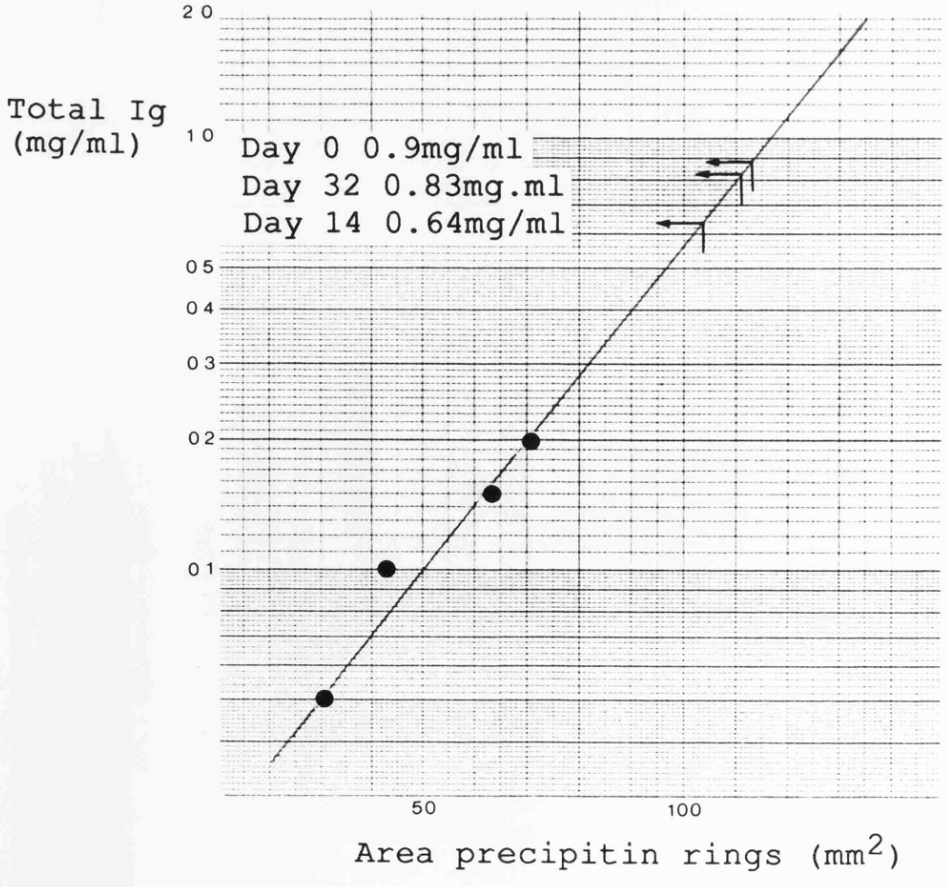
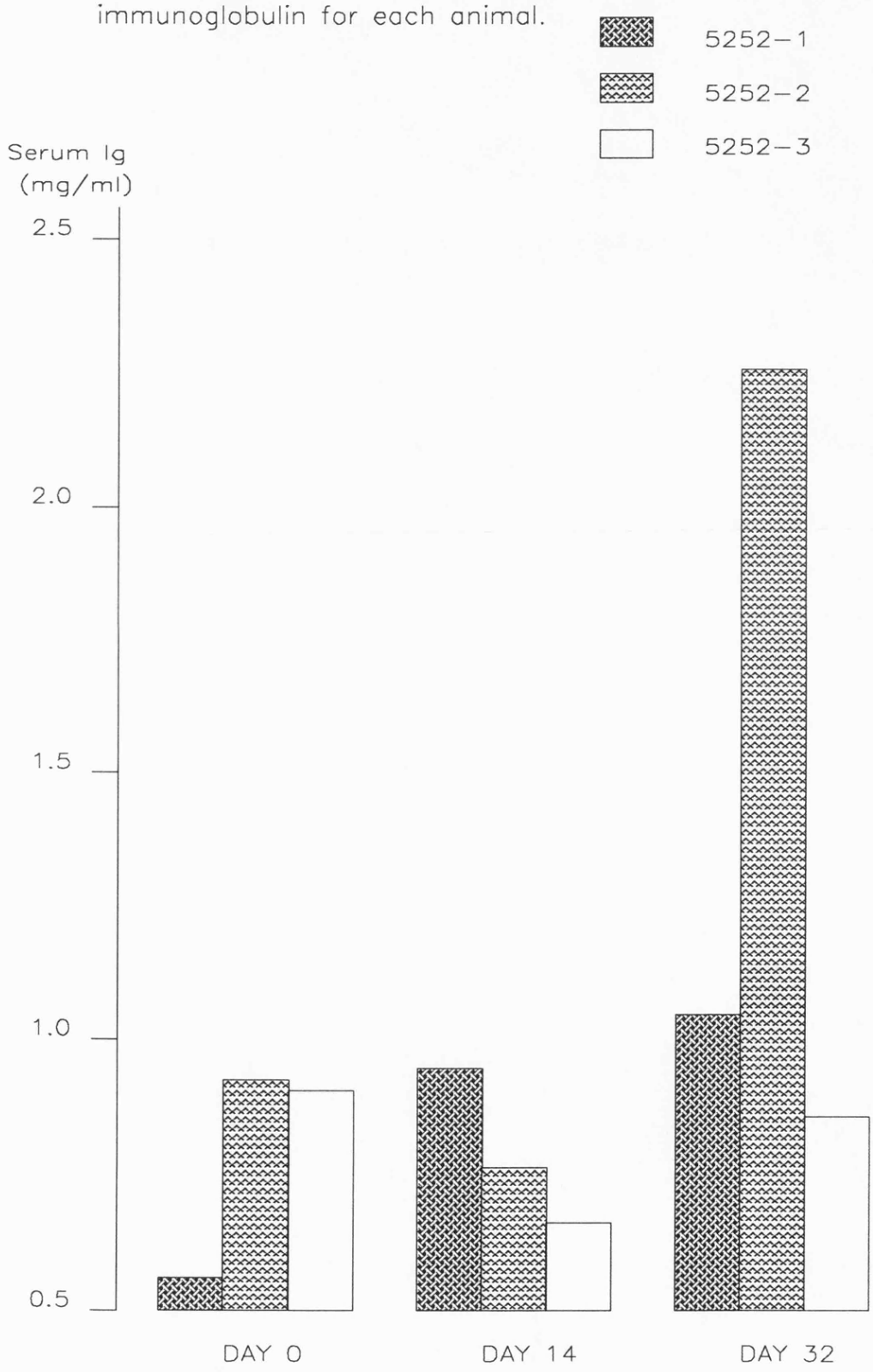


Figure 4.2c. Time course graph showing total rabbit immunoglobulin for each animal.



to four wells on each slide. To the six remaining wells on each slide serum samples from day 0, day 14 and day 32 were added in duplicate. Wells were filled quickly until the meniscus just disappeared and the slides were incubated at room temperature for 48hrs. Slides were washed in PBS overnight at 37°C then dried at 45°C. The precipitin rings were stained using Coomassie Brilliant Blue R250 as described for electrophoresis gels in section 2.3.7, and destained until the background was clear (figure 4.2a). The diameter of the precipitin rings was measured. From the calibration standard curves (figure 4.2b) the mean total Ig present in the serum of each animal at day 0, day 14 and day 32 was determined (figure 4.2c).

Measurement of the total immunoglobulin levels in the rabbit's serum was done as a measure of the immunologic responsiveness of each animal to the immunogen. The scarcity of the glycoprotein precluded its use as an antigen in assaying for the antibody.

4.2.5. Affinity purification of P5252.

Attempts were made to affinity purify the polyclonal antiserum by nitrocellulose adsorption (Olmsted, 1981; Krohne *et al.*, 1982). Concentrated and dialysed culture medium derived from a breast carcinoma known to contain the glycoproteins of interest, was electrophoresed and blotted. A duplicate sample and standard lanes were stained with amido black (see section 2.4.) to locate the position of the appropriate glycoproteins on the unstained

nitrocellulose. These regions (about 5mm x 3mm) were cut out, blocked with BSA (see also section 2.4.) and incubated with the polyclonal antiserum for 3 hours at room temperature. The blot was washed in PBS for 30 minutes and the antibody eluted by incubation for 5 minutes at room temperature with 3M KSCN PBS (pH 6.0-6.5). Immediately after this incubation PBS was added to dilute the KSCN to a 1M solution, which was then dialysed and concentrated using the centrifugation technique described earlier (section 2.3.1.), to yield a final volume of 100-150ul. It is important that the potassium thiocyanate is diluted and dialysed as quickly as possible since prolonged exposure would denature any protein present.

With hindsight the technique of dialysis used was inappropriate since it both dialysed and concentrated simultaneously. However this was known at the time and steps were taken to minimize the initial stages of concentration by interrupting centrifugation and diluting with fresh PBS. Nevertheless it is likely that this was insufficient in preventing irreversible damage to the eluted antibody.

The adsorption of non-specific antibody activity onto acetone washed spleen powder was attempted as a means of partially purifying the polyclonal antiserum (Nairn, 1976). Bovine spleen acetone powder was wetted with PBS, centrifuged at 10000xg for 15 minutes and the supernatant discarded. The polyclonal antiserum was added (total protein, 100mg/ml) and gently stirred to dislodge the pellet.

The mixture was agitated gently for 2 hours at room temperature and centrifuged at 10000xg for 15 minutes and the supernatant removed. The supernatant was compared with unadsorbed P5252 on protein blots (section 4.3.3 and figure 4.3) and tissue sections using the indirect peroxidase technique described below (section 4.3.1.).

4.3. Immunological Applications.

4.3.1. Immunohistochemistry.

A majority of the immunohistochemistry work done involved the indirect immunoperoxidase method. All incubations were at room temperature unless otherwise stated. A number of tissues including breast were used in these studies, all of which were fixed in 4% formaldehyde for between 18 and 48 hours and processed as previously described (section 2.1.2.). Paraffin sections cut at 4um onto glass microscope slides were dried overnight at 37°C before dewaxing in xylol and rehydrating through descending alcohols. For some initial studies parallel sections were incubated for 30 minutes at 37°C in a 0.1% trypsin solution containing 0.1% $CaCl_2$ before blocking any endogenous peroxidase activity by further incubating in a 0.3% H_2O_2 solution in methanol for 30 minutes. After washing in tap water, distilled water then PBS, the tissue sections were blocked with normal swine serum (serum of the same species as the secondary antibody)

at 1/5 dilution for 10 minutes. The slides were drained of this blocking solution (but not washed) and the primary antibody, P5252, applied at 1/200 or 1/500 dilutions (11.25 or 4.5mg/ml total rabbit Ig) for 2 hours. After two 5 minute washes in PBS the peroxidase conjugated secondary antibody, swine anti-rabbit, was applied at 1/50 dilution for 45 minutes. Further washes in PBS preceeded the visualization steps using DAB for 7 minutes (see section 2.4.3.). After rinsing in running tap water the sections were counterstained for 20 seconds in Meyers Haematoxylin, washed in running tap water and rinsed in Scott's solution for 20 seconds. After washing thoroughly in running tap water the sections were dehydrated through ascending alcohols to xylol and mounted in XAM mounting medium.

The exposure to trypsin is sometimes necessary to permeabilize the tissue sections to obtain better accessibility of the antibody to its specific binding site. This is dependant on a number of factors including fixation of tissue and antigen location. It was found that there was little difference between those incubations with and without trypsin and this step was therefore omitted in later studies.

4.3.2. Competition studies.

In order to assess the specificity of the polyclonal antibody, P5252, in relation to other selected antibodies which could detect similar epitopes, competition studies were performed. The protocol was similar to that described above (section

Table 4.1. Protocol outline for competition studies. (See text, section 4.3.2). Primary antibodies; HMFGII, BRST1 and P5252. Peroxidase labelled secondary antibodies; Sw-Rb= swine anti-rabbit, Rb-Mo= rabbit anti-mouse.

SLIDE SET No.									
1	2	3	4	5	6	7	8	9	10
HMFGII BRST1	P5252	HMFGII BRST1	P5252	HMFGII BRST1	P5252	-	-	HMFGII BRST1	P5252
P5252	HMFGII BRST1	-	-	-	-	-	-	P5252	HMFGII BRST1
Sw-Rb	Rb-Mo	Rb-Mo	Sw-Rb	Sw-Rb	Rb-Mo	Sw-Rb	Rb-Mo	Rb-Mo	Sw-Rb
TEST A (reverse of B)	TEST A	POSITIVE CONTROLS		CROSS-REACTION CONTROLS		NEGATIVE CONTROLS		TEST B (reverse of A)	TEST B

4.3.1.) but including where appropriate, a competing primary antibody. For simplicity the protocols are outlined in table 4.1. Incubation times and dilutions were the same as for the indirect peroxidase method unless stated otherwise. Competition studies were performed between the following antibodies: P5252 and HMFG2; P5252 and BRST-1 (at 1/20 dilution).

4.3.3. Immunoblotting with P5252.

Protein blots were prepared as described in chapter 2, and probed with HMFG2 antibody, fibronectin antiserum and the polyclonal antiserum P5252. The antibody titres and peroxidase labelled secondary antibodies are listed below;

HMFG2	1/20	Rb-Mo
Anti-fibronectin	1/100	Sw-Rb
P5252	1/200	Sw-Rb

Rb-Mo= Rabbit anti-mouse immunoglobulin antiserum (peroxidase labelled).
Sw-Rb= Swine anti rabbit immunoglobulin antiserum (peroxidase labelled).

4.3.4. Radio-labelling of secondary antibodies for P5252.

The swine anti-rabbit immunoglobulin antiserum was radiolabelled with ^{35}S in order to increase the sensitivity of detection on immunoblots as well as reducing the background staining due to the DAB staining protocol (2.4.3.). The labelling procedure used a commercial sulpher labelling reagent, SLR. A

200ul aliquot of swine anti-rabbit antiserum was lyophilized overnight and redissolved in 0.1M borate buffer (pH 8.5). To a siliconized tube 400uCi of SLR, in toluene, was added and allowed to evapourate to dryness. The dissolved lyophilate was then added and incubated for 30 minutes. The reaction was stopped by the addition of 400ul of 0.2M glycine in 0.1M borate buffer (pH 8.5) and lyophilized. The lyophilate was finally redissolved in PBS and used at 1/50, 1/100 and 1/200 dilutions. The conjugated antibody was tested on a protein blot of a breast carcinoma sample known to release the glycoprotein 230 and 250.

The secondary antibody was labelled, and not the primary (P5252), so as to maintain the sensitivity of the indirect method. One of the major disadvantages of using a labelled primary antibody (as in the direct method), is the decreased sensitivity, compared to the indirect method where the secondary antibodies are labelled. The latter is more sensitive since at least two labelled secondary antibodies can bind to each primary antibody molecule thereby amplifying the final signal.

4.4. Results

4.4.1. Antibody production.

The fluorescent screening technique showed negligable non-specific activity of the pre-immunized rabbit serum to human breast tissue when compared with

Figure 4.3. Western blot of proteins released from a grade III infiltrating ductal carcinoma showing labelling with untreated P5252 (A) and spleen powder adsorbed P5252 (B).

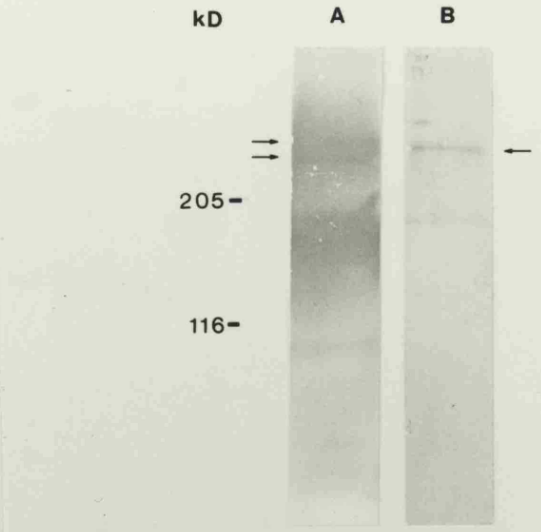


Figure 4.4. Fluorogram of a Western blot of proteins released from a grade III infiltrating ductal carcinoma showing labelling with untreated P5252. Visualized by ^{35}S conjugated swine antiserum to rabbit immunoglobulins.

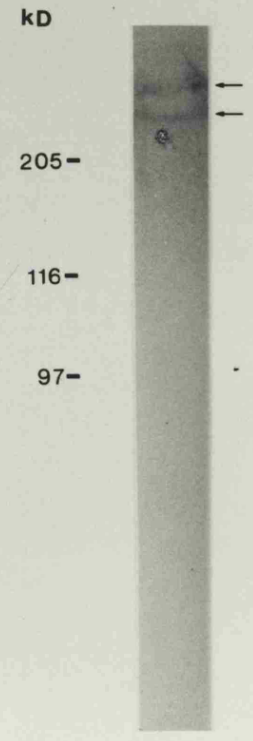


Figure 4.5. Western blot of proteins released from malignant cultured breast tissue, showing binding of HMFG II (lane A) and P5252 (lane B).

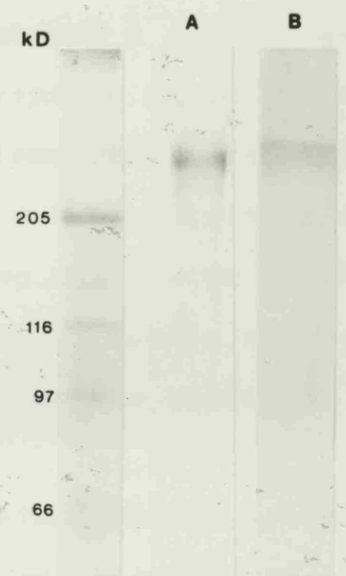
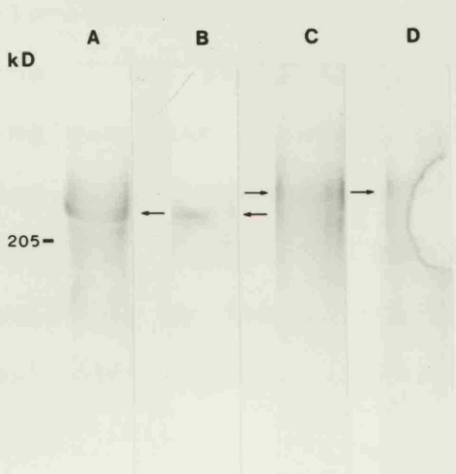


Figure 4.6. Western blot of proteins released from two malignant cases, showing binding of anti-fibronectin antibody (lanes A and B) and P5252 (lanes C and D).



polyclonal antibodies to secretory piece and fibronectin.

The total serum immunoglobulin levels for each animal are shown in figure 4.2c. Only one animal, rabbit 2-5252, showed a significant elevated immunological response as determined by the increased level in total immunoglobulin. All animals remained healthy with core temperatures maintained between 38°C and 39.4°C during the course of the experiment. Both hind legs were fully mobile throughout and there were no visible lumps at the injection sites.

4.4.2. Purification, characterization and labelling.

Affinity purification of the antisera showed a marked reduction in antibody titre, as determined by immunohistochemistry on selected breast tissues. The spleen powder adsorbed P5252 when tested on a Western blot showed binding to only one band above 205kD and faint binding to a band at about 190-200kD as compared to the untreated P5252 (figure 4.3). Although the specificity was increased the titre was markedly reduced.

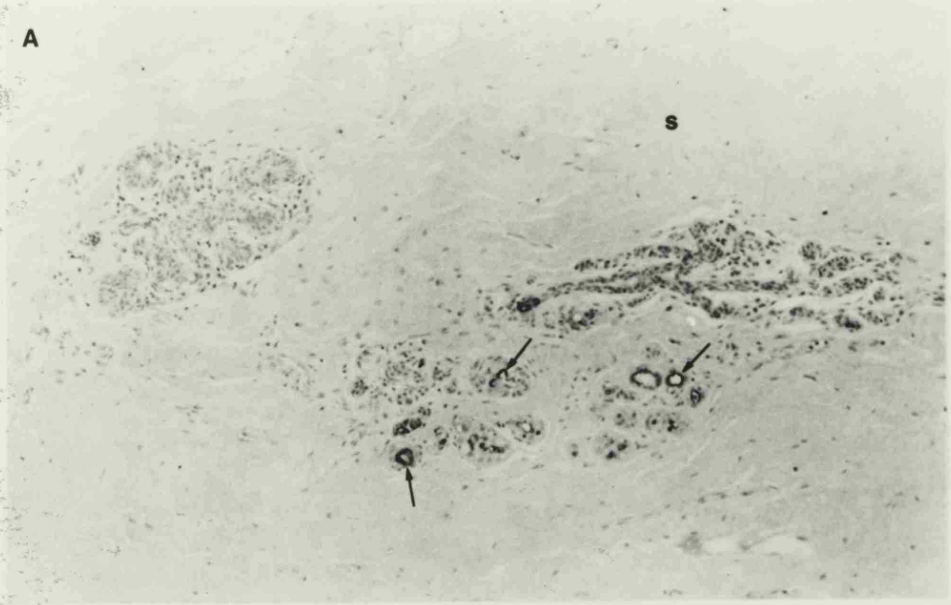
The ^{35}S radio-labelled antibody complex showed binding to two bands at 230-250kD (figure 4.4). Although the binding was weak it was still specific to these high molecular weight components.

The comparisons of P5252 with HMFG2 and anti-fibronectin antibody by immunoblotting show binding to components of slightly different mobility (figure 4.5 and 4.6).

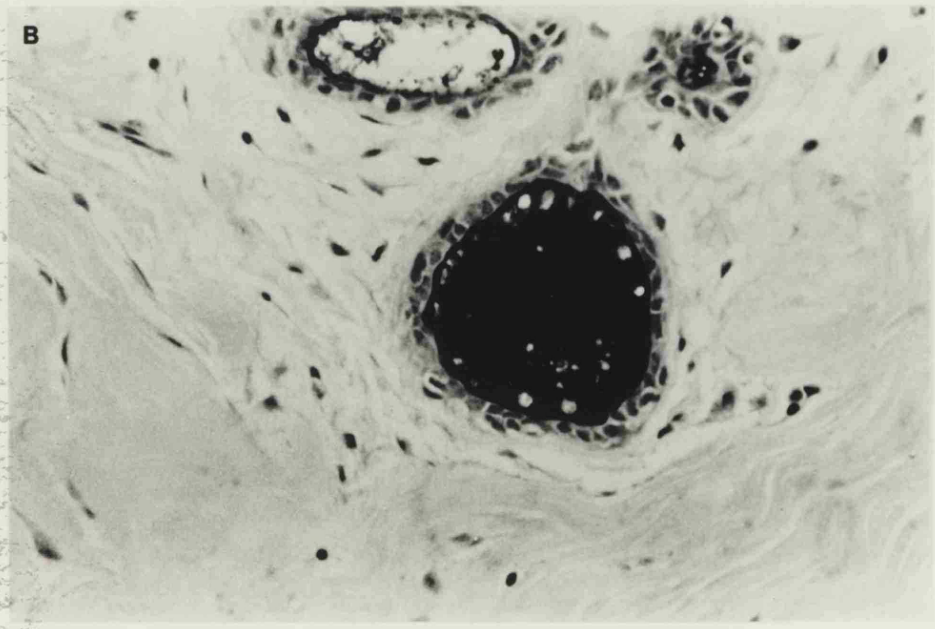
Table 4.2a Tissue types studied using polyclonal antibody P5252.

Tissue type		No. cases Normal	Tumour
G.I. tract			
	Salivary gland	1	-
	Oesophagus	1	-
	Stomach	5	6
	Liver	1	-
	Appendix	1	-
	Large intestine	6	6
G.U. tract (female)			
	Ovary	3	6
	Fallopian tube	6	-
	Uterus; endometrium	2	2
	myometrium	2	-
	cervix	5	-
G.U. tract (male)			
	Testis	1	1
	Vas Deferens	1	-
	Prostate	1	1
Endocrine			
	Adrenal	-	1
	Breast	19	59
	Thyroid	1	1
Other			
	Skin	1	3

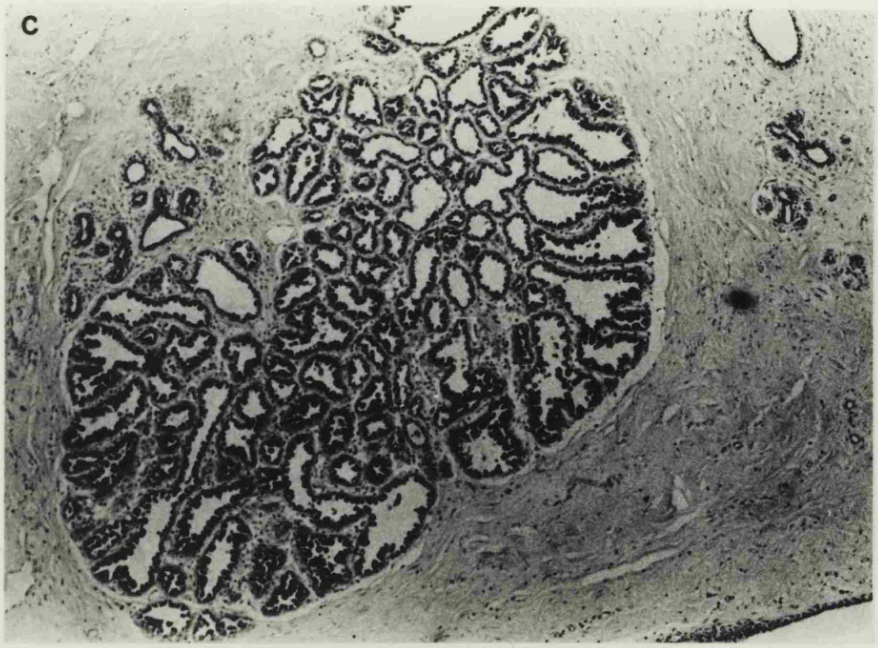
Figure 4.7. Reactivity of The Polyclonal Antiserum P5252 with Tissue Sections of Human Breast.



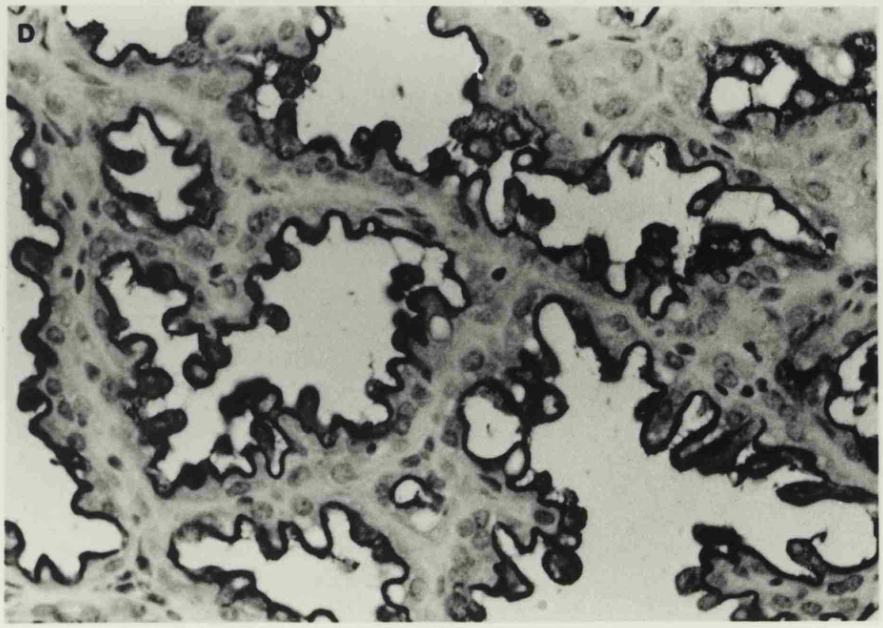
A: Normal breast, with one lobule showing no evidence of reactivity and two adjacent lobules showing luminal staining of a small number of acini (arrowed); s=stromal tissue.



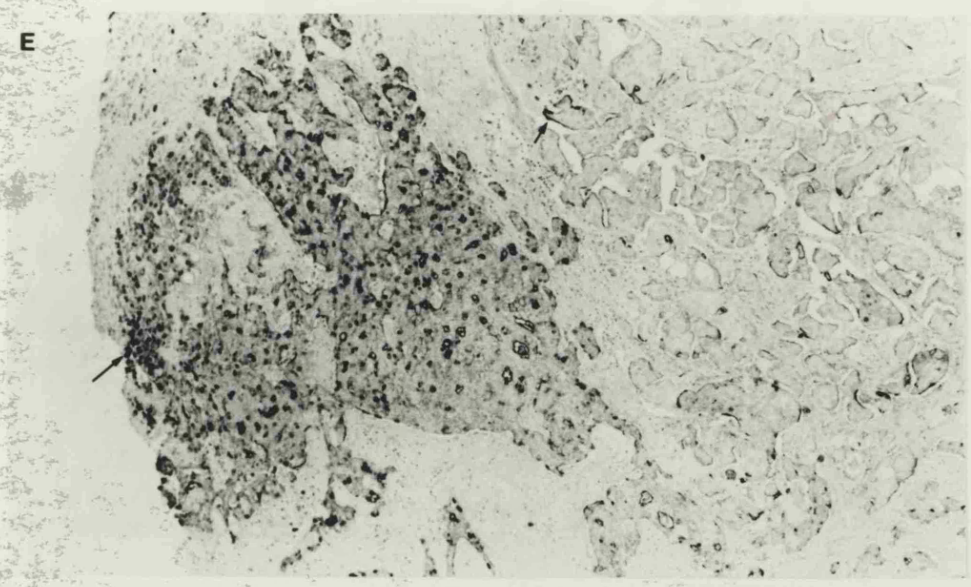
B: Different non-malignant breast sample at high magnification to demonstrate luminal membrane and glandular secretions staining.



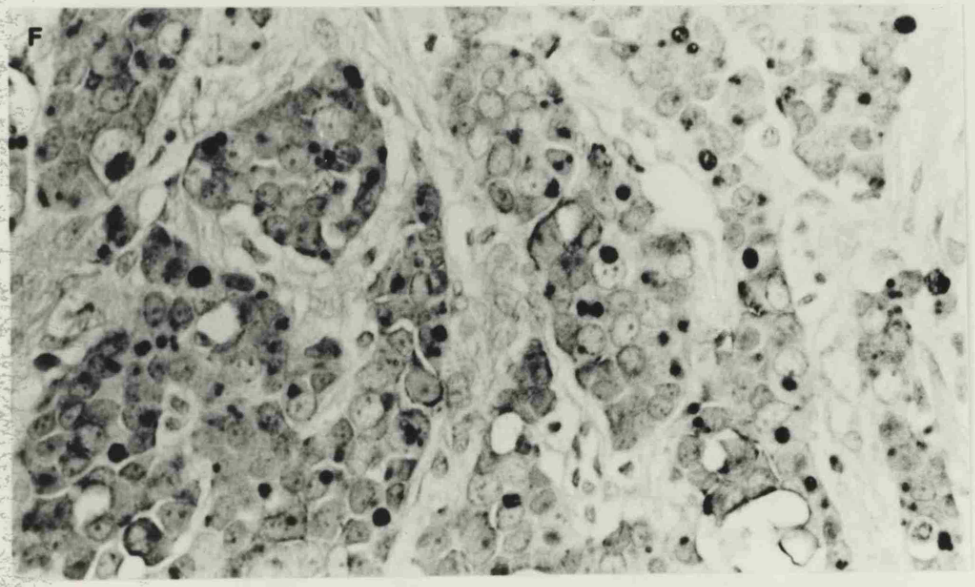
C: Pregnant breast lobule showing staining of luminal surface.



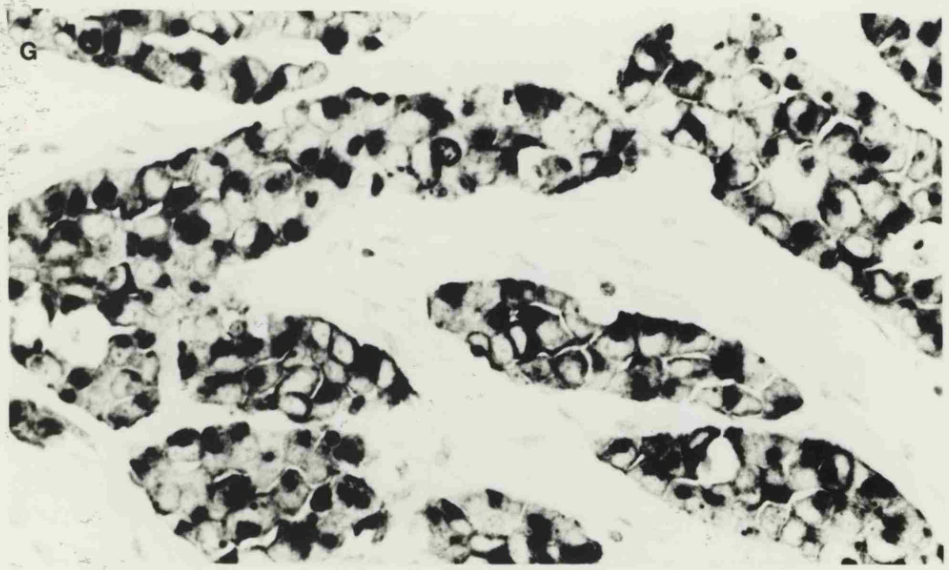
D: As C, but at higher magnification. Note clear staining of luminal borders, with minimal cytoplasmic reactivity.



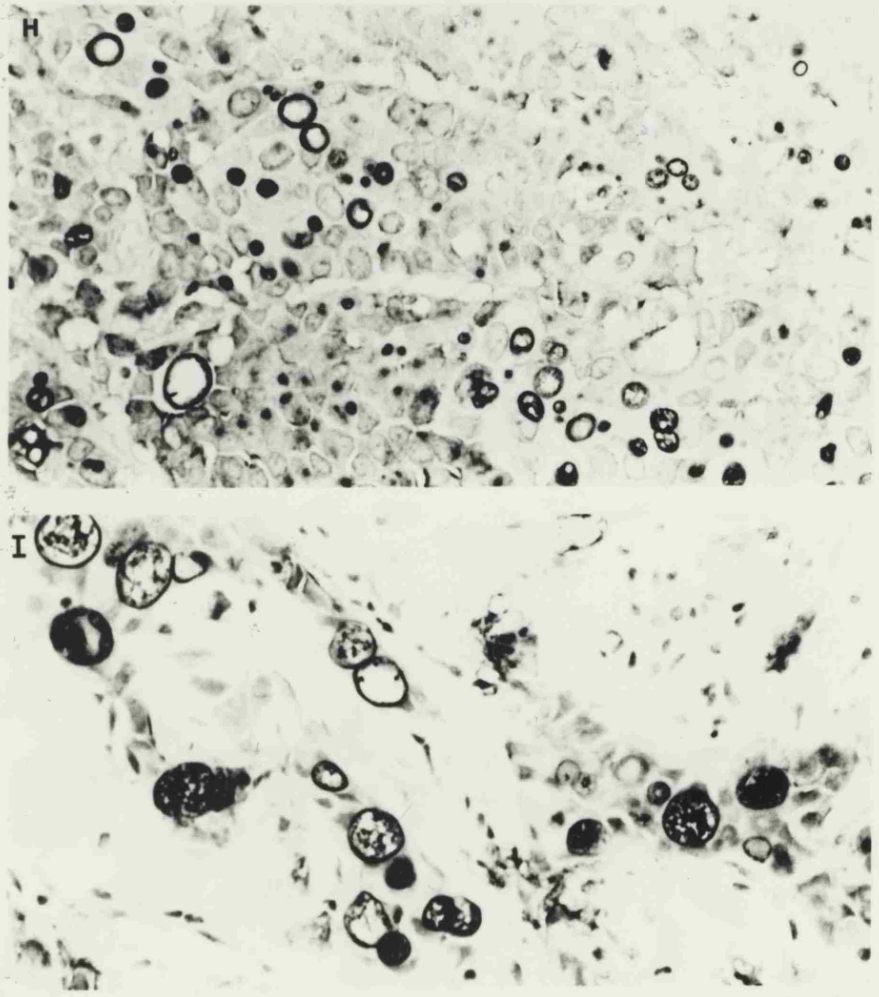
E: Breast carcinoma demonstrating heterogeneity in the proportion of reactive cells and in the localization. Note staining of luminal surface (short arrow) and focal cytoplasmic staining (long arrow)



F: As E, but at higher magnification showing predominantly focal cytoplasmic, with some ICL and cell surface reactivity.

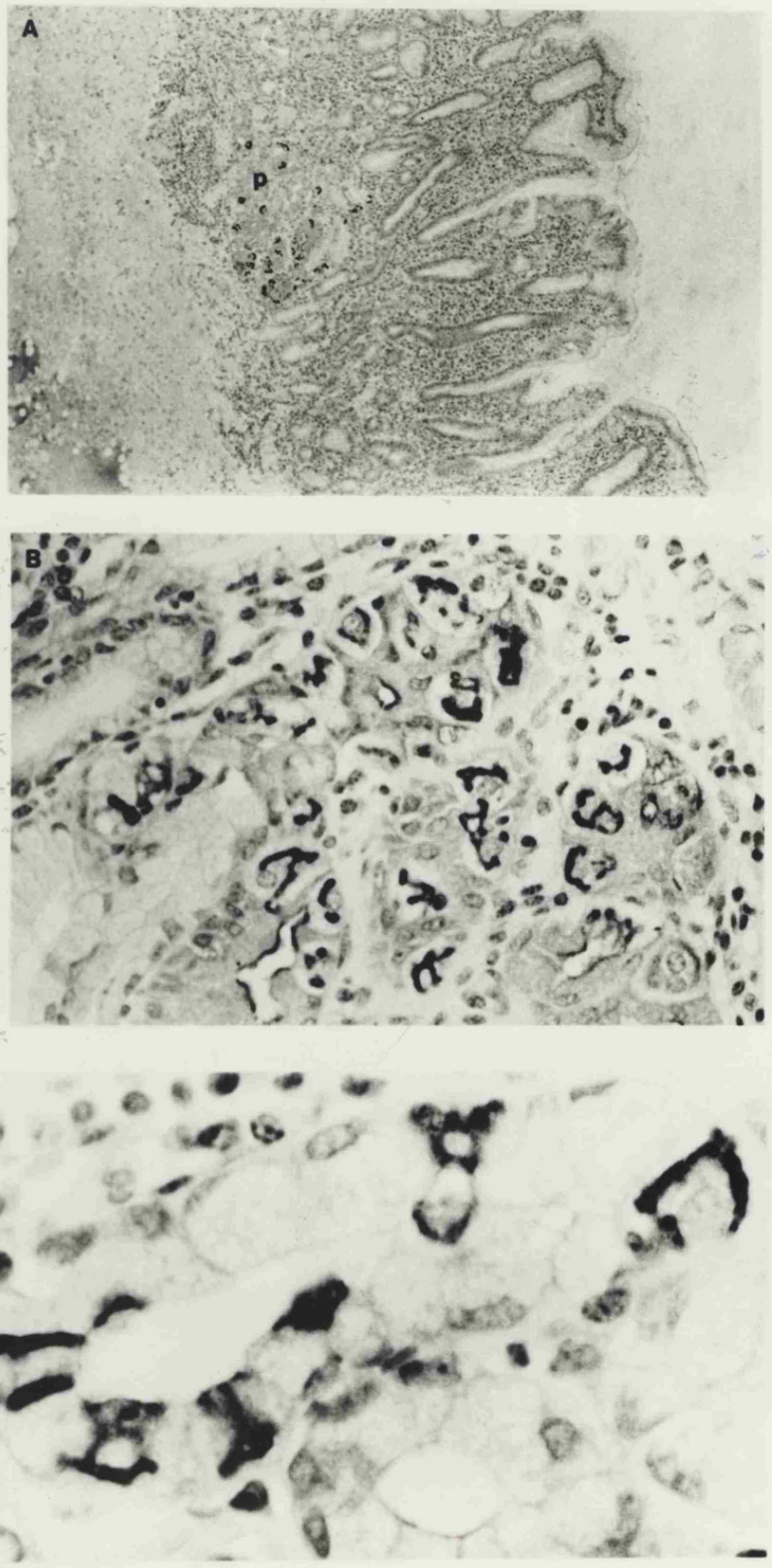


G: A different carcinoma in which the staining is predominantly focal and diffuse cytoplasmic.

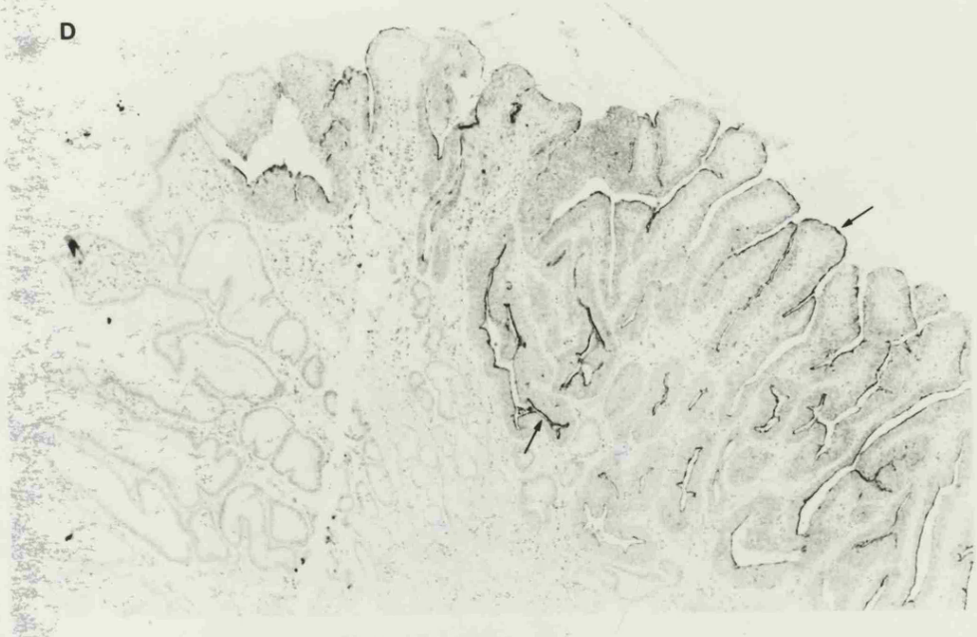


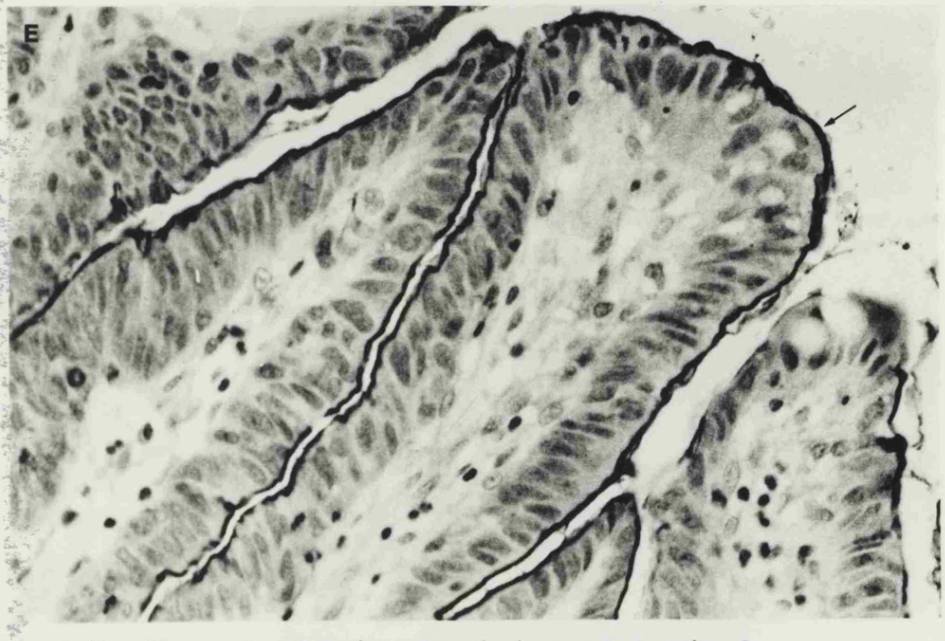
H and I: Two seperate carcinomas in which the predominant site of staining is of intracytoplasmic lumina.

Figure 4.8. Reactivity of The Polyclonal Antiserum P5252 with Tissue Sections of The Stomach.



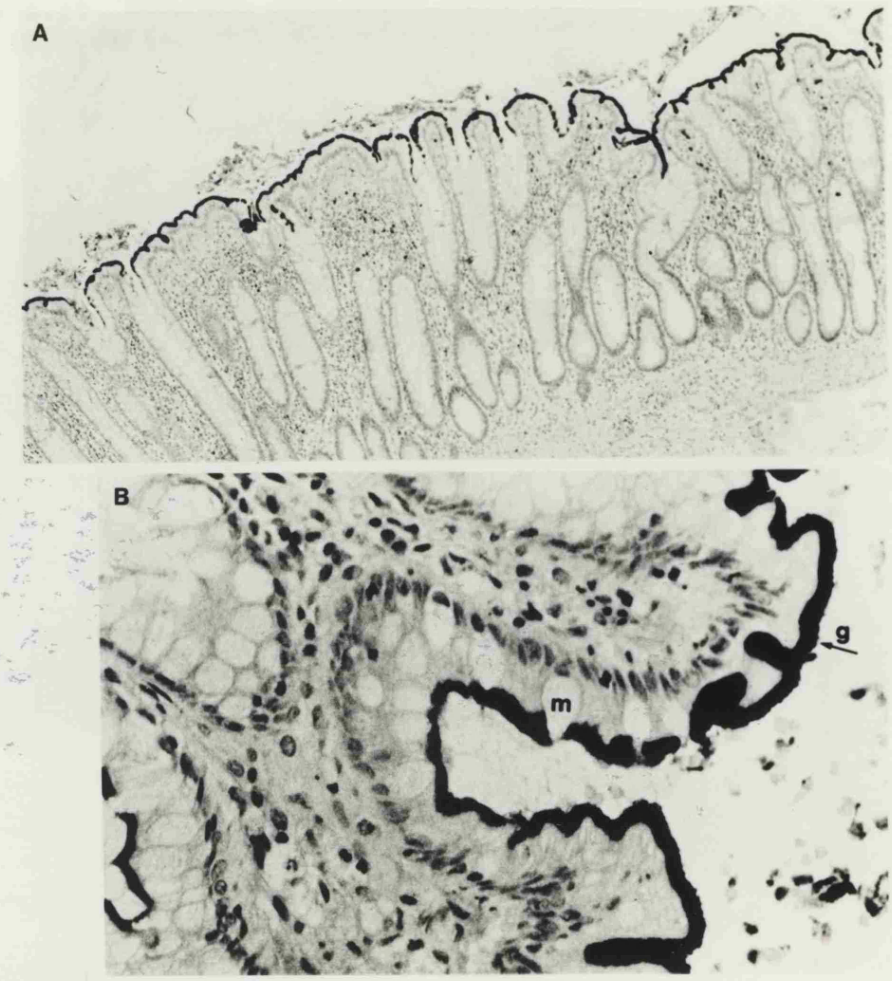
A, B and C: Sections of normal stomach showing staining in a group of parietal cells (p). Note localization of stain in the cannaliculi.



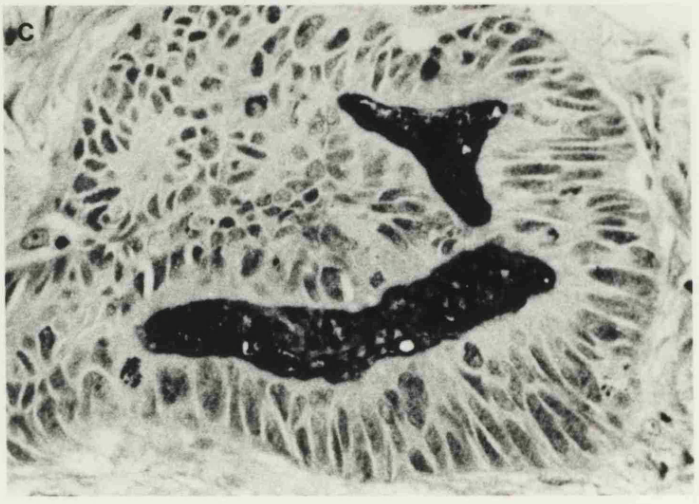


D and E: Stomach showing staining of luminal border/glycocalyx only in areas of carcinoma-in-situ (arrowed)

Figure 4.9. Reactivity of The Polyclonal Antiserum P5252 with Tissue Sections of The Large Intestine.



A and B: Normal tissue from a patient with rectal adenocarcinoma showing strong staining of the glycocalyx (g). Note the absence of staining of mucin secreting cells (m).



C: Carcinoma of ascending colon showing staining of glandular secretions.

Figure 4.10. Ovarian carcinoma showing reactivity with P5252 in some but not all areas of tumour.

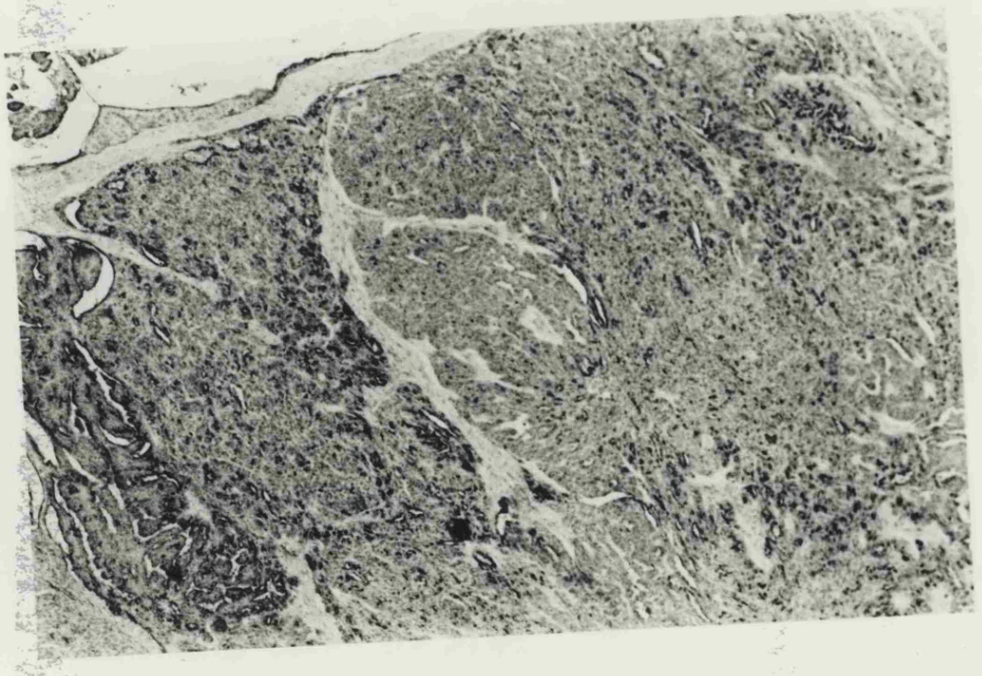
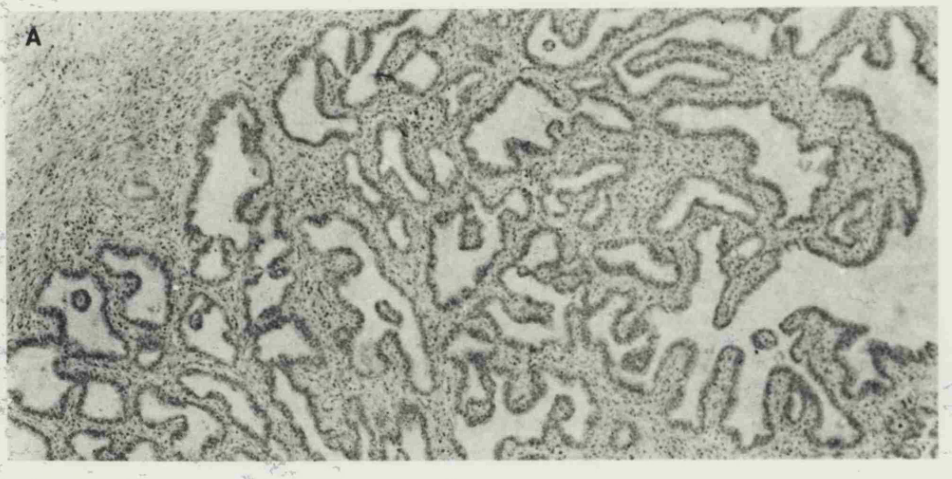
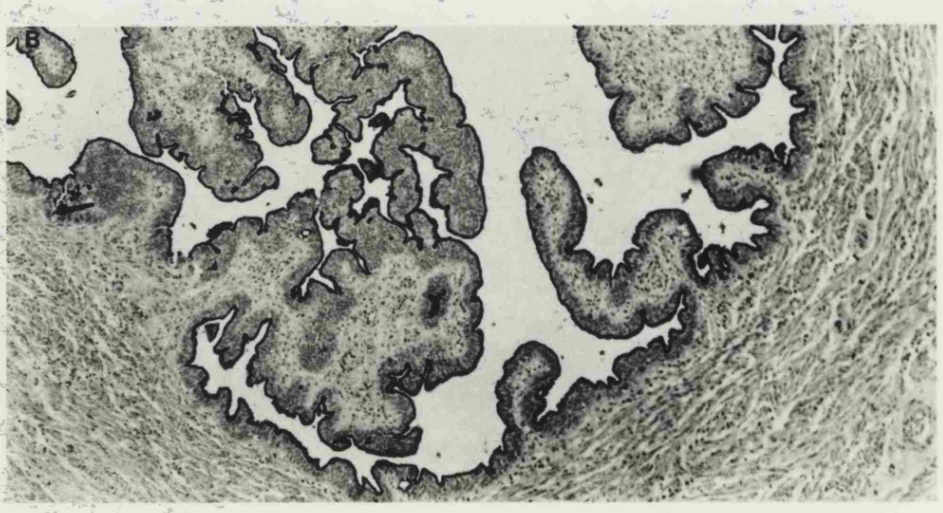


Figure 4.11. Reactivity of The Polyclonal Antiserum P5252 with Tissue Sections of Fallopian Tube.



A: Normal tissue showing no staining.



B: Normal tube epithelium showing staining of luminal borders in fallopian tube with endometriosis and inflammation.

C: As B, but at higher magnification.

Figure 4.12. Endometrial tissue with signs of cystic hyperplasia showing reactivity with P5252 at the luminal borders of the glands.

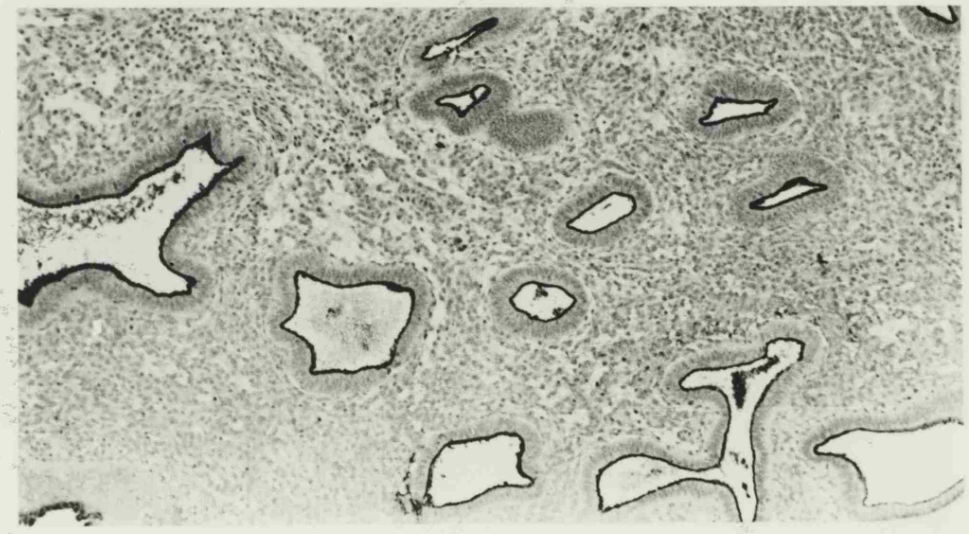
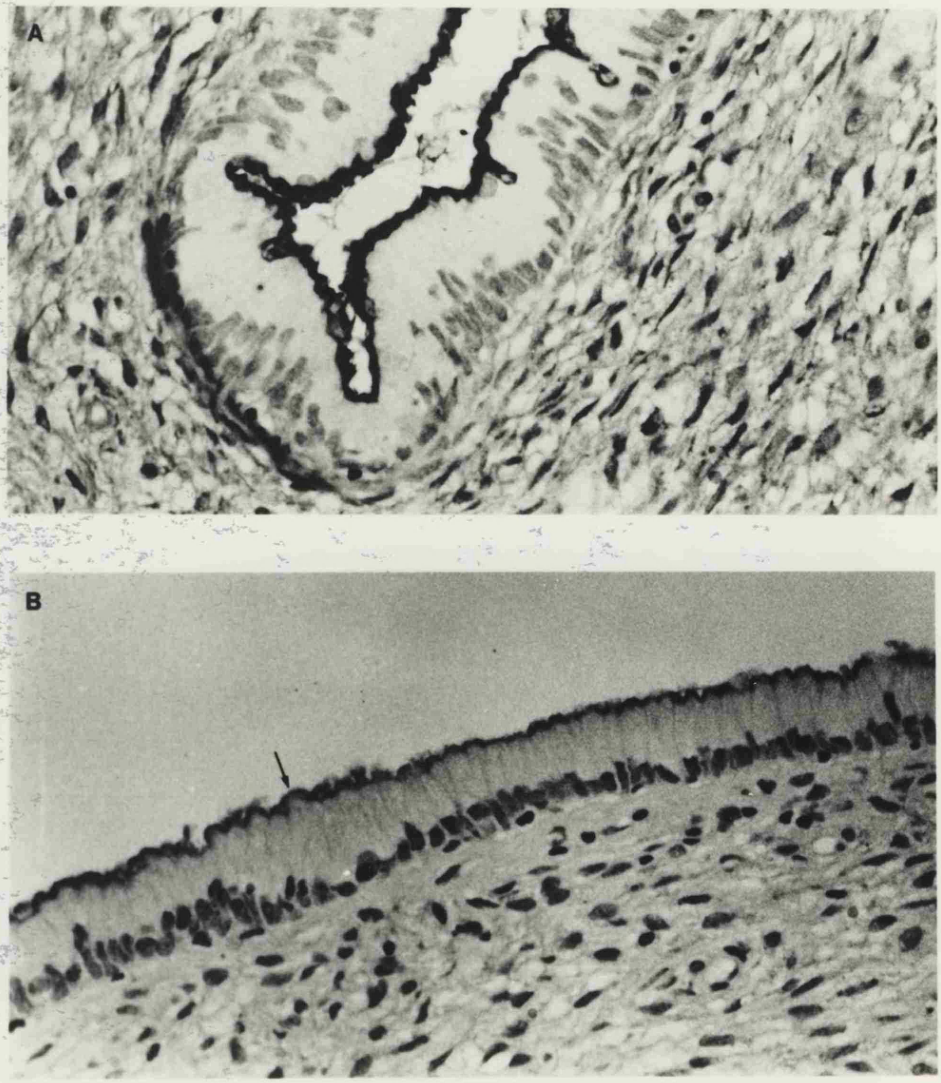
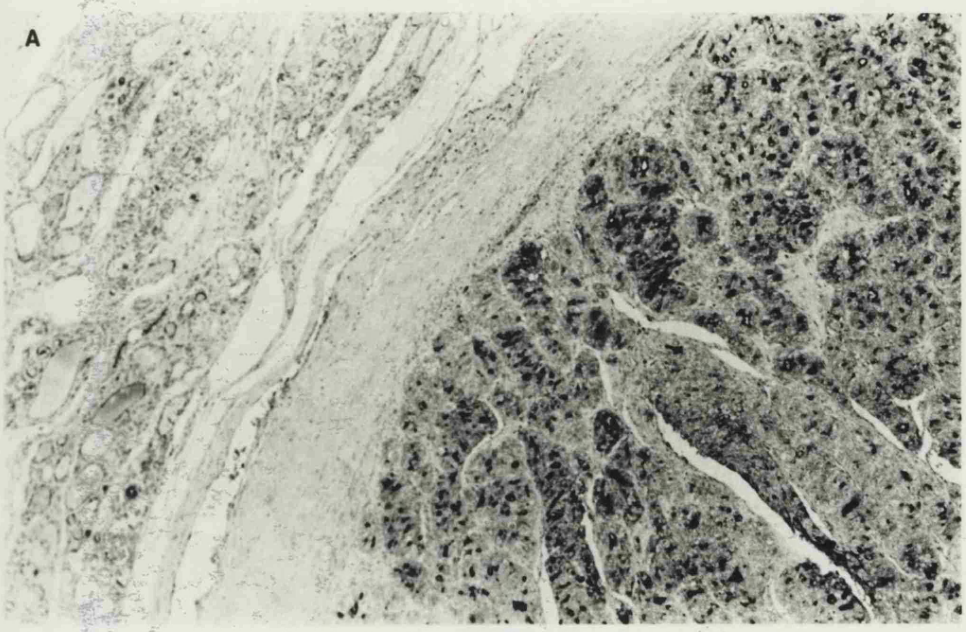


Figure 4.13. Reactivity of The Polyclonal Antiserum P5252 with Tissue Sections of Endocervix.

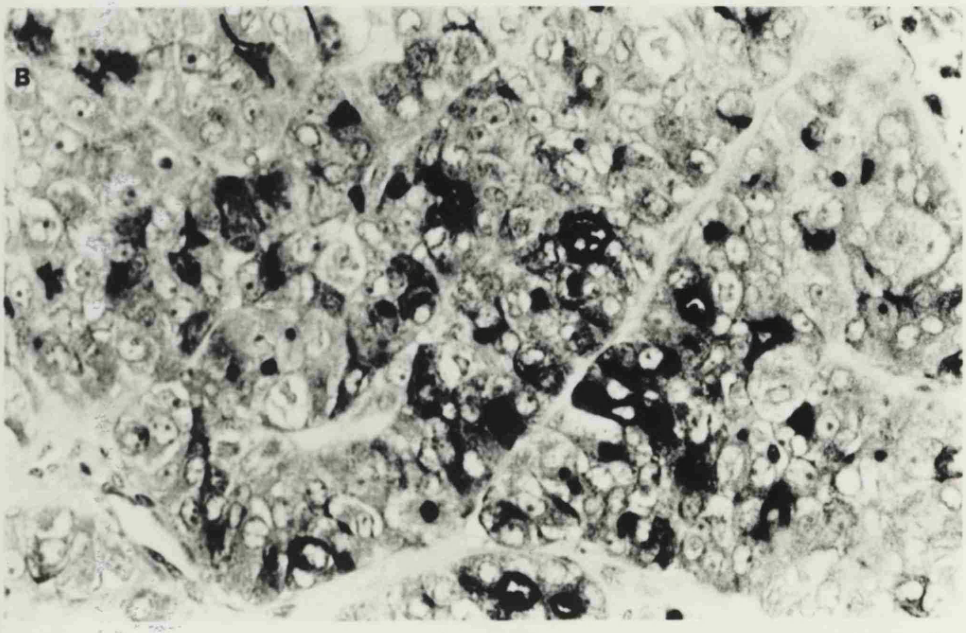


A and B: Surface and glandular epithelium of the endocervix showing staining of the luminal borders.

Figure 4.14. Reactivity of The Polyclonal Antiserum P5252 with Tissue Sections of Thyroid Carcinoma.

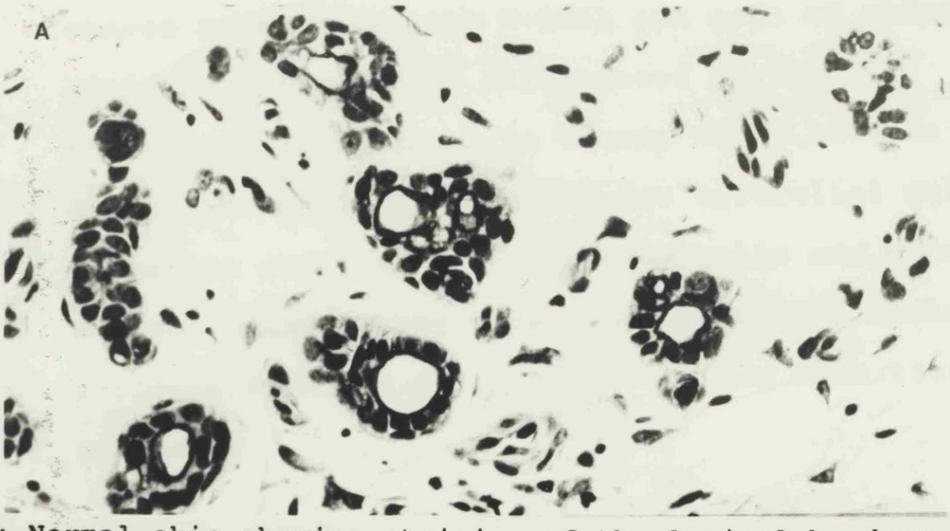


A: Follicular carcinoma (right of photograph) showing strong staining of tumour cells, with adjacent normal thyroid being essentially negative.

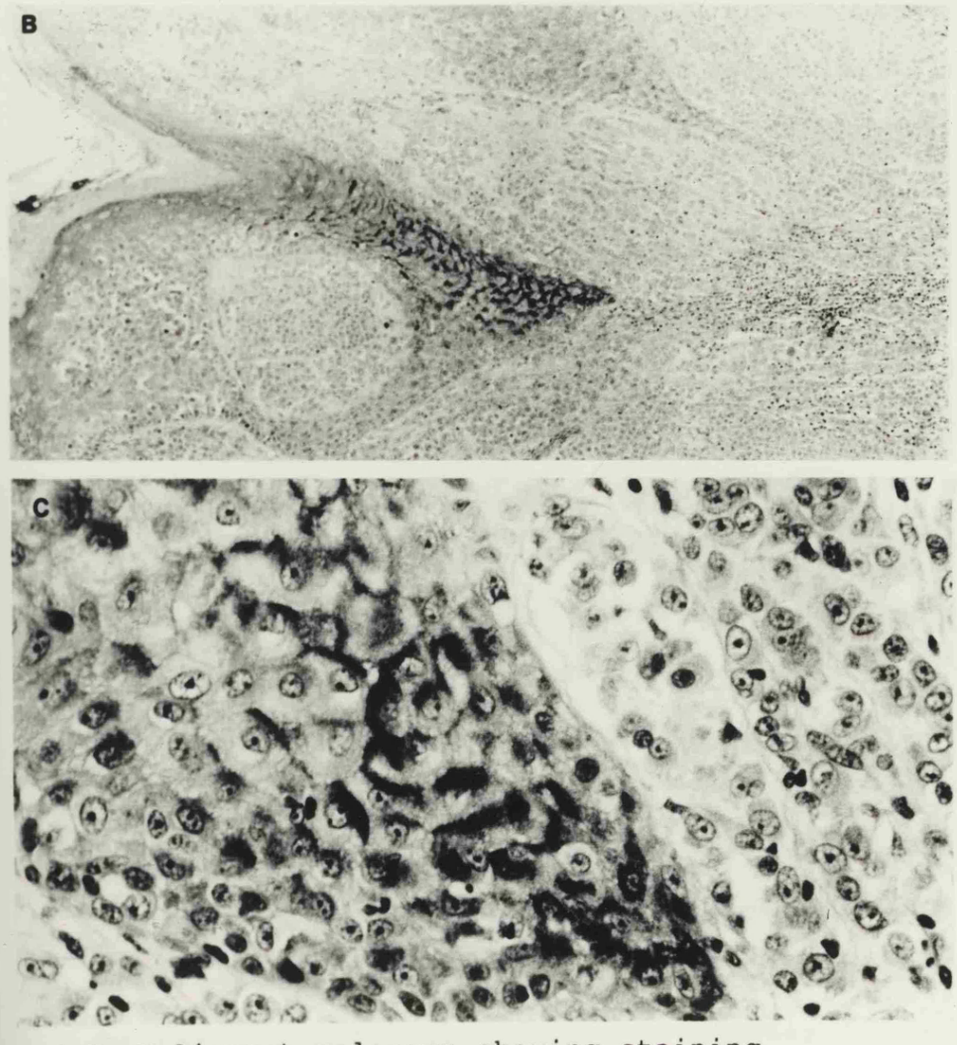


B: As A, but at higher magnification. Note strong focal and cytoplasmic staining.

Figure 4.15. Reactivity of The Polyclonal Antiserum P5252 with Tissue Sections of The Skin.



A: Normal skin showing staining of the luminal borders of the merocrine glands.



B and C: Malignant melanoma showing staining predominantly of oneside of the cell of stratified squamous epithelium adjacent to underlying melanoma cells.

4.4.3. Immunohistochemistry.

A number of the epithelial tissues studied showed reactivity with P-5252 and some of these staining patterns are illustrated in the photomicrographs shown in figure 4.7-4.15. The antiserum P5252 was specific for epithelial cells and showed no reactivity with plasma cells and other stromal cells of any type. The individual staining patterns seen are described below for each tissue studied.

Breast:

Benign and normal areas of tissue showed reactivity of P5252 in the acini lumens with not all the acini staining (figure 4.7.a). Cytoplasmic staining was negligable. Some glands showed staining of "secretory" products (figure 4.7.b). Reactivity of the antiserum in benign and normal areas was less intense, with fewer cells staining, than malignant specimens. Lactating breast also showed marked luminal staining of active lobules (figure 4.7.c and 4.7.d). In malignant tissue the staining pattern was markedly altered, with many tumours showing distinct focal cytoplasmic staining (figures 4.7.e to 4.7.g). Many malignant cells expressed intracytoplasmic lumina (ICL's) which also stained strongly on the luminal borders (figure 4.14.h and 4.7.i). The ICL structures were predominatly in tumours showing poorer differentiation. Reactivity of the antiserum in most of the malignant specimens was greatly increased, both

Table 4.2b. Comparison table of biochemical and immunohistochemical data for some of the carcinomas studied. F= fucose, G= galactose. (*= no fucose data).

CASE No.	% POSITIVE CELLS	GLYCOPROTEIN EXPRESSION 210	230	250	280
470	75	-	+F	+G	-
472	10	+G	+F	+F	+G
475	40	+FG	+FG	-	-
479	0	-	+F	+G	-
480	50	+FG	-	+G	+FG
502	70	+FG	-	+G	+FG
507	75	+FG	+FG	-	+G
510	60	+FG	+F	-	+FG
515	80	+F	+FG	-	-
522	95	-	+F	-	-
529	30	+F	+G	-	+G
535	0	+FG	-	+FG	+F
543	10	+FG	-	+FG	+F
547	95	+FG	-	+FG	+FG
601	75	+G	+FG	-	-
644*	80	+G	-	-	+G
647	40	-	+FG	-	+G
648	25	-	+G	+FG	+G
651	75	+FG	-	+F	+G

Table 4.2c. Summary table relating the percentage of reactivity of carcinomas with P5252 with histological differentiation and node status, where known. Neg= negative node status, Pos= positive node status.

	PERCENTAGE OF CELLS STAINING 0	<50	>50
Grade:			
I	0	1	2
II	1	10	12
III	1	12	7
Node:			
Neg	1	6	8
Pos	0	14	8

in intensity and percentage of cells staining, than that observed in the benign and normal areas of tissue (table 4.2b and 4.2c).

Gastro-intestinal tract:

Oesophagus. No staining was observed in normal oesophagus.

Stomach. In normal stomach, staining was confined to parietal glands (figure 4.8.a), the cannaliculi of which showed a marked staining reaction (figure 4.8.b and 4.8.c). Tumour tissue however showed staining of the glycocalyx, which was particularly pronounced in a case of carcinoma in situ (figure 4.8.d and 4.8.e), and of carcinoma cells to varying degrees.

Liver. No staining was observed in normal liver.

Appendix. No staining was observed in normal appendix.

Large intestine. A variety of staining patterns were seen in this tissue. Only one of four rectal adenocarcinomas showed reactivity with P5252 which appeared to be localized to glandular secretions. In the normal tissue from the same patient there was a strong reaction in the glycocalyx of the surface epithelium, with mucin secreting cells not staining (figure 4.9.a and 4.9.b). Normal tissue excised from the ascending colon showed no reactivity with the antiserum although the malignant tissues from the same site showed staining of luminal membranes plus secretions (figure 4.9.c).

Genito-urinary tract (female):

Ovary. No staining was observed in sections of normal

ovary. Two of the six cases of ovarian carcinoma showed strong focal cytoplasmic and luminal staining (figure 4.10.a) in 70% of epithelial cells.

Fallopian Tube. No staining was observed in sections of normal fallopian tube (figure 4.11.a). In two cases where endometriosis was present there was strong staining of the glycocalyx of both the normal epithelium and the endometriosis, (figure 4.11.b and 4.11.c).

Endometrium. There was no staining in one case of normal endometrium in the proliferative phase. Endometrium showing signs of cystic hyperplasia showed staining which was localized to the luminal membrane (figure 4.12). Two adenocarcinomas showed focal and diffuse cytoplasmic staining.

Myometrium. No staining was observed in normal myometrium.

Cervix. The luminal membranes of normal endocervical glands were strongly reactive with the antiserum (figure 4.13.a and 4.13.b). Faint cytoplasmic staining was observed in some cases, although this was weak and heterogenous. Weak staining was also observed at the ecto/endocervical transition zone for one case which also showed signs of inflammation.

Genito-urinary tract (male):

Testis. No staining was observed in either normal testis or seminoma.

Vas Deferens. Faint and heterogenous staining of luminal membrane cells.

Prostate. No staining was observed in prostatic

hyperplasia.

Endocrine tissues:

Adrenal Gland. No staining was observed in normal adrenal and adrenal carcinoma.

Thyroid. Heterogenous staining of follicular epithelium was observed. The staining although heterogenous in intensity was specific for follicular epithelium (figure 4.14.a), with 90% of tumour cells staining. The reactivity was predominantly focal and cytoplasmic (figure 4.14.b) with some luminal borders of ducts staining. Occassional staining was observed in normal areas, although the frequency of cells staining increased nearer the site of malignancy.

Other tissues:

Skin. No staining was observed in the normal areas of keratinized epithelium. There was strong luminal membrane staining in the cells of the merocrine glands (figure 4.15.a). In one case cytoplasmic staining was detected in the sebaceous gland. A case of malignant melanoma showed marked granular staining only of those squamous epithelial cells adjacent to underlying melanoma cells (figure 4.15.b and 4.15.c). This staining was more pronounced in some areas than others.

The reactivity of the antibodies HMFG2, BRST-1 and P-5252 in the competition studies are summarized in table 4.3. There was no cross reactivity between the mouse monoclonals and the secondary peroxidase labelled antibody (slide set no. 5). There was also no

Table 4.3. Staining reactions for competition studies. See also table 4.1. for corresponding protocol outline. (+= positive staining, -= no staining).

Key: Slide set no. 1. Shows staining with P5252 after incubation with HMFG II or BRST1.
2. Shows staining with HMFG II or BRST1 after incubation with P5252.
3. Positive control for HMFG II or BRST1.
4. Positive control for P5252.
5. Controls showing cross reaction between mouse monoclonals HMFG II and BRST1, and swine anti-rabbit secondary antibody.
6. As 5 but between rabbit polyclonal P5252 and rabbit anti-mouse secondary antibody.
7 and 8. Negative controls.
9 and 10. As 1 and 2 but reversing the order of application of the primary competing antibodies.

	SLIDE SET No.									
	1	2	3	4	5	6	7	8	9	10
HMFG II										
RW458	++	+	+	++	-	-	-	-	+	++
RW473	++	++	+	+++	-	-	-	-	+	++
RW507	+	+	+	++	-	-	-	-	+	++
RW547	+++	+++	+++	+++	-	-	-	-	+++	+++
BRST1										
RW458	++	+++	+++	++	-	-	-	-	++	+++
RW473	+++	+	+	+++	-	-	-	-	+	++
RW507	++	++	+	++	-	-	-	-	++	+++
RW547	++	+++	+++	+++	-	-	-	-	+++	+++

cross reactivity between the rabbit polyclonal antiserum and the rabbit anti-mouse immunoglobulin antiserum (slide set no. 6). The 'no-primary' controls also showed no staining (slide set no.'s 7 and 8). The positive controls (slide set no.'s 3 and 4) where no competition was permited, showed staining patterns of similar intensity to the test or competition slide sets (no.'s 1,2,9 and 10). The order in which the competing antibodies were applied to the tissue section did not appear to affect the final staining pattern for P5252 (slide set no.'s 1 and 10) and HMFG2 or BRST-1 (slide set no.'s 2 and 9).

4.5. Discussion.

The marked reduction of antibody activity observed after the purification methods attempted, prompted the use of whole antisera in the subsequent immunohistochemical studies. This reduced antibody activity was probably due to the random non-specific adsorption of protein to the acetone washed spleen preparation, thereby proportionally reducing the amounts of available antibody (Nairn, 1976). It was subsequently found that acetone powders can contaminate antisera with soluble tissue constituents, although not as badly as freeze dried powders, and that fresh tissue homogenates often yield the best results. Some loss of antibody titre must be expected if only from random physical adsorption of

immunoglobulin molecules. Other methods of antisera purification often involve the exposure of antibodies to low pH, and/or extremes of salt concentrations, which adversley affects their stability. This reduced antibody stability is probably the result of hydrophobic interaction between the IgG molecules in solution. Moreover the increased hydrophobicity of their aggregates is likely to cause increased nonspecific binding in immunohistochemistry. Exposure to mild treatment such as ion-exchange chromatography was not done, since there was subsequently found to be minimal background stromal staining observed by immunohistochemistry. This was unexpected since there were a number of bands detected on the Western blots with P5252 (figure 4.3. lane A). However, after detection with the ^{35}S labelling technique the same untreated antiserum preparation showed that only two bands were visible. The extra binding components observed by Western blotting could be due to other antibodies present at much lower affinity. The reduced sensitivity seen with the ^{35}S labelled antiserum may be due to disruptive effects of the SLR reagent on the antibody's antigen binding domains. The binding of the reagent to free amino-groups may have sterically interfered with the antiserum binding activity at the N-terminus of the variable light chain (V_L).

The presence of two bands in the untreated antiserum is notable in light of the preliminary findings from peptide mapping of the glycoproteins of 230 and 250kD. It is not unreasonable to expect that

the apparent homology between these two glycoproteins is reflected in their antigenicity. However, as the purity of the inocculum was only checked by 1-D and not 2-D PAGE, it remains a possibility that the additional band at 250 kD is due to insufficient separation of the two glycoproteins in the antigen preparation schedule.

Immunohistochemical studies using the antiserum P5252, showed reactivity only with epithelial cells, with no activity in plasma cells or other stromal cells of any type. This is contrary to the staining patterns seen with the HMFG antibodies, which detect epitopes in plasma cells. This supports the findings of the competition studies that P5252 and HMFG2 detect different epitopes. Reactivity with the antiserum P5252 was not apparent in many histologically normal non-breast tissues; oesophagus, liver, appendix, ovary, fallopian tube, myometrium, testis, adrenal gland, and skin. These findings were similar to a previous study reviewing the activity of a number of antibodies raised against HMFG and other breast antigens recently designated human polymorphic epithelial mucins (PEM's; Taylor-Papadimitriou, 1988). The weak and occassional staining in normal and benign breast; with many acini and ducts showing no reactivity with P5252, was similar to the staining patterns seen with the monoclonal antibodies M26 and B72.3, (Zotter, *et al.*, 1988). Similar to other antibodies directed against breast antigens, P5252 reacted strongly with lactating breast (Zotter, *et*

al., 1988). Indeed of all the monoclonal antibodies to breast tumour antigens and MFGM, only B72.3 is negative with lactating breast (Nuti, *et al.*, 1982).

The faint staining of P5252 in normal and benign breast tissue contrasted with the strong staining observed in most of the carcinomas studied, highlighting a significant quantitative difference. In the malignant breast specimens, in particular those showing poor differentiation, staining was localized focally in the cytoplasm and when present was associated with intra cytoplasmic lumena (ICL's). These large vacuole-like structures in the cytoplasm are variable in size and often large enough to displace the nucleus. It is likely that the reactivity of P5252 in these structures is a result of a "mis-targetting" of membrane or secretory components originally destined for the plasma membrane. This altered "targetting" of surface or secreted components in the malignant state, is not a novel phenomena, and has been described using other markers, e.g. the MAM series (Hilkens, *et al.*, 1984).

The reactivity of the antiserum only in part parallels the biochemical findings of chapter 2; the quantitative elevation of expression of the glycoprotein of 230kD is particularly marked in malignancy. However the reactivity in benign tissue, although much weaker, does not support the expression of the glycoprotein of 230kD as determined by the methods described in chapter 2. This may be due to the structural similarities, discussed earlier, between

the glycoproteins of 230 and 250kD, the latter showing expression in some benign samples (table 2.3). The same effects however would be seen if, as also discussed earlier in this chapter, the antigen preparation contained both glycoprotein components.

The reactivity of P5252 in non-breast epithelial tumour tissue showed some significant staining patterns. The reactivity of P5252 with squamous epithelium adjacent to melanoma may be associated with the malignancy. The reactivity was specific to these areas and was not the result of non-specific "binding" to keratinizing areas, which can occur due to insufficient washing in the immunohistochemical procedure. Moreover, the staining pattern with P5252 is similar to that observed for a polyclonal antiserum against epithelial membrane antigen (EMA), in inflammatory lesions in squamous epithelium (Sloane & Ormerod, 1981).

In the tumour tissue of the stomach there was a strong reactivity of the antiserum in the glycocalyx. This was most pronounced in the case of carcinoma *in situ* shown in figures 4.8.d and 4.8.e. In areas of normal stomach taken both from the same and different patients, only the parietal cells showed rectivity. The staining in these cells appeared to be localized in the intracellular cannaliculi. These structures are considered to be part of the secretory apparatus of the cells and represent an infolded cell surface covered by numerous microvilli. The staining of parietal cells in this fashion is indicative of a

secretory or membrane associated component. This pattern of staining is similar to a number of monoclonal antibodies raised against the HMFG determinant; HMFG2, M8, 115D8, and E29, and other breast cancer antigens; B72.3, F36/22, 139H2, 140C1 and NCRC11 (Zotter, *et al.*, 1988).

The staining patterns observed in the large intestine did not correlate with the UEA-I binding sites in non-neoplastic mucosa of human colon and rectum as described by Suguru *et al.*, (1982). Indeed mucin secreting cells did not react with the antiserum P5252. This binding pattern was again similar to that seen with NCRC11, as mentioned above. The absence of UEA-I lectin binding correlation supports earlier findings from chapter 2 that UEA-I showed only weak reactivity with the high molecular weight group of glycoproteins, in particular the glycoprotein of 230/250kD.

The staining patterns in the tissues studied, in particular the breast, stomach and large intestine, suggests that the antiserum P5252 reacts with a tumour-associated antigen with epitopes similar in distribution, to those detected on human PEM (Taylor-Papadimitriou & Gendler, 1988). A number of tissues still require further analysis to complete the immunohistochemical study of the tissue and tumour distribution of the epitopes detected by the antiserum P5252. In addition the assessment of the cellular localization of these epitopes by electron microscopy would provide more information regarding the

distribution and possible function of the P5252 determinant.

CHAPTER 5

ANALYSIS OF BREAST CELL LINES.

The problems experienced with the small amounts of tissue available from carcinomas provoked investigation for a model system of *in-vitro* culture. The two systems considered were: a) primary cell culture; and b) existing breast cell lines. The former system could provide cultures of cells closely related to the original tumour. The ability to grow these epithelial cultures directly from individual primary tumours would be a great asset. The heterogeneity within individual tumours could be maintained and many cell-cell interactions would not be selected for or against, since growth would only be taken up to the first passage. The problems encountered with dense stromal tissue and poor cellularity in organ culture could be avoided. Manipulations, e.g., in preparing sub-cellular fractions, would be greatly simplified with higher yields obtained.

The use of breast cell lines would provide an unlimited supply of tumour cells for analysis. This

would enable research to continue in the absence of a regular supply of primary tissue, to permit analysis of cell behaviour under specific conditions, and allow many other cell manipulations. Furthermore the heterogeneity seen between breast carcinomas, although a notable feature, can complicate certain aspects of study.

Although preliminary studies showed a small degree of success in isolating and maintaining primary cultures, the chapter concentrates on the technically easier of the two options considered; the analysis of established human breast cell lines. Human breast cancer cell lines constitute a valuable tool for the study of breast cancer with both human and animal cell lines being used in a variety of biochemical, immunological, genetic and virological approaches. In this study the metabolically labelled glycoproteins released during the culture of each cell line was determined, with particular emphasis on the high molecular weight glycoproteins identified in chapter 2.

The expression of the group of high molecular weight glycoproteins was compared with the reactivity of the polyclonal antiserum P5252, (chapter 4) in cytospin preparations of the same cell lines.

Initial attempts to purify sufficient quantities of the glycoproteins for subsequent deglycosylation, peptide mapping, and carbohydrate and protein sequencing analysis was unsuccessful, the reasons being discussed in section 5.7.

The work outlined in section 5.1. was part of a poster presented at the 158th meeting of the Pathological Society of Great Britain and Ireland (see appendix G). Section 5.4. was also part of a poster presented at the X^{th} International Symposium on Glycoconjugates (see appendix G).

5.1. Cell Culture From Primary Tumours.

5.1.1. Dissaggregation of tissue.

The major development of these techniques was done by Dr. D. Corcoran and will form a substantial part of his own PhD thesis. For this reason only the methods specifically used by the author will be described, and subsequent discussion of the development of these techniques will be limited.

Enzyme disaggregation. Primary tumour tissue was diced

finely into 1mm cubes as described for organ culture (see section 2.2.1.). No pre-incubation period was necessary and tissue was cultured as for the raft organ culture technique but using 13-15 explants in each dish. The culture medium (1:1 DMEM/Ham's F12, v/v), in addition to the fungizone and antibiotic supplements, contained 0.5mg/ml hyaluronidase, 0.5mg/ml collagenase, and 0.25mg/ml DNAse. The cultures were incubated in an atmosphere of 5% CO_2 and 95% air for up to 48 hours. The time of this incubation period varied depending on the stromal content of individual tumours. The disaggregated tissue was teased off the lens paper into the medium and the larger clumps of tissue disaggregated further by passing in and out of a 21g needle four or five times. The cell suspension, excluding the very large clumps, was centrifuged at 400xg for 5 minutes and the supernatant carefully removed. After a wash with fresh culture medium (without enzymes) and further centrifugation, the pellet was finally resuspended in primary culture medium (see section 5.1.2.).

Mechanical disaggregation. This technique for dissociating epithelial cells from the surrounding connective tissue was used only on those tumours which immediately appeared very cellular. This was noticed not only by the decreased resistance to the blades but also by the cloudy fluid released when cutting the tissue into cubes. When tumours of this type were found, the fluid released from the cutting was incubated directly in the primary culture medium (see

section 5.1.2.) along with the diced cubes of tumour tissue.

5.1.2. Primary cell culture.

Cells produced from either of the disaggregation protocols were resuspended in primary culture medium which comprised 5ml DMEM/Ham's F12 (1:1), 5ug/ml fungizone, 100ug/ml penicillin and streptomycin, 5% foetal calf serum (FCS), 10ug/ml insulin, 1ug/ml hydrocortisone, 0.5mM dbcAMP and 1.0mM theophylline. This cocktail was added to a small cell culture flask ($25cm^2$) to provide a high cell density, and left undisturbed at 37°C in 96% air, 5% CO_2 for 3-4 days. After this "settling" period the medium was removed and fresh medium added with reduced concentration of dbcAMP (0.25mM) and theophylline (0.5mM). Cultures were maintained with a bi-weekly medium change until about 70% confluent whereupon subculture was necessary (usually day 20-25). Cells were subcultured using a variety of methods; rubber policeman (mechanical), trypsin/EDTA (enzymatic), 5mM disodium EDTA (chemical) or 20mM lignocaine (chemical). Post sub-culture growth was supported by fresh DMEM/Ham's F12, containing filtered conditioned medium (1:1 v/v) from the initial culture. In this limited series of experiments cell growth after sub-culture was not achieved.

5.2. Cell Lines.

5.2.1. History of cell lines.

All cell lines described were generously donated by ICI Pharmaceuticals and were lines obtained from the American Tissue Culture Collection (ATCC); BT20 (Lasfargues and Ozzello, 1958), BT474 (Lasfargues *et al.*, 1978), HS578T (Hackett, 1977), MDA MB-231 (Cailleau, *et al.*, 1974), MDA MB-468 (Cailleau, *et al.*, 1978), T47D (Keydar, *et al.*, 1979), HBL100 (Gaffney, 1976), and MCF-7 (Soule, *et al.*, 1973). Of these cell lines only three were derived directly from primary tumours; BT20, BT474 and HS578T, the remaining cell lines, with the exception of the HBL100's, were obtained from metastases, usually from pleural effusions. The HBL100 cell line was derived from the milk of a lactating mother three days after delivery. Although there was no evidence of a breast lesion and no familial history of breast cancer, the cell line was determined to have an abnormal karyotype determined at passage 7.

The HBL100 and BT474 cell lines were obtained at a later date and were consequently only included in the immunohistochemistry study.

5.2.2. Receipt and storage protocols.

All procedures involved with the preparation and handling of cells were performed in a Class II laminar flow cabinet. Incubations were at 37°C in an atmosphere of 5%CO_2 and 95% humidity. Upon receipt, cell lines were immediately subcultured and incubated with fungizone, penicillin and streptomycin, in

addition to any other individual requirements. Antibiotic and fungizone concentration was gradually reduced and eventually omitted, allowing at least 4 clear days before sub-culturing. Cell lines were maintained with bi-weekly medium changes until confluent. Cells from each $80cm^2$ flask were removed by adding 1ml of a trypsin/EDTA solution and agitating for 1-2 minutes. When most of the cells had rounded-up and detached, determined by monitoring with phase contrast microscopy, 4ml of the balanced salt solution (BSS) was added and the resulting cell suspension centrifuged at 400xg for 5 minutes. The supernatant was carefully decanted and the cell pellet resuspended by flicking the tube and slowly adding 1ml DMEM (added dropwise over 1 minute to prevent the cells from clumping). These cells were reseeded into as many $80cm^2$ flasks as their individual plating efficiency permitted. The procedure was repeated to further expand the stocks of cells ready for freezing. Before freezing the cell lines, the contents of each $80cm^2$ flask was subcultured as described above up to the point of adding the 1ml of DMEM. The cell suspension was then recentrifuged and finally resuspended in 1ml of "freezing medium"; DMEM containing 10% DMSO, 10% FCS. This high cell density suspension was placed in a cryotube and stored overnight at -70°C and finally placed in the vapour phase of liquid nitrogen until required for use. For the purposes of laboratory records these frozen aliquots of cell lines were given the notation P_0 and labelled as "primary stock". Each

subsequent passage of these freezer stocks was then labelled P_1,P_2,... and so on, indicating the number of passages or sub-cultures each cell line has been through. Initial expansion of this "primary stock" (see also section 5.4.1.) was necessary to provide a seperate "working stock" of which all subsequent cell line experiments would use.

5.3. Immunohistochemistry With P5252.

5.3.1. *Preparation of cytospins.*

Cells were grown to confluence and subcultured as described (section 5.2.2.). Instead of reseeding further cultures the 1ml cell suspension in DMEM was used to make a cytospin preparation. A cell count using an haemocytometer was done and the cell suspension diluted to yield $4x10^5$ cells/ml, 250ul of this was centrifuged onto dimethyldichlorosilane coated microscope slides, fixed in 10% formalin for 10 minutes and stored (up to 1 week) in PBS at 4°C until required.

As an alternative to cytospin preparations commercial chambered slides (Lab-Tek) were used. Cells from each line were added to the eight individual wells on the slide and allowed to adhere and grow to 80% confluence (2-3 days). After incubation the medium was drained and the cells fixed in 10% formalin for 10 minutes. The plastic chambers were removed, rinsed in PBS then processed in parallel with the cytospins for

immunochemical localisation of the antibody P5252.

5.3.2. Immunohistochemistry protocols.

Cytospin and slide culture preparations were incubated with the antibody P5252 according to the indirect peroxidase method described in section 4.3.1. and subsequently visualized using the DAB method (also section 4.3.).

5.4. Analysis of Medium Released From Cell Culture.

5.4.1. Cell culture; procedure and conditions.

Vials of six of the cell lines (MCF7, HS578T, T47D, BT20, MDA MB231 and MDA MB468) from primary freezer stocks were removed from liquid nitrogen storage and thawed rapidly by placing in a covered plastic beaker at 37°C. When thawed the contents of one vial was added to one 80cm^2 flask with the appropriate growth medium and maintained until there was a confluent monolayer. The cells were sub-cultured and reseeded into two 25cm^2 flasks and at least two 80cm^2 flasks (depending on plating efficiency). The latter cultures were maintained, subcultured and eventually frozen, labelled as "working stock" and used in all subsequent cell line experiments. The two small flasks were maintained until 70% confluent when the medium was removed and discarded. The monolayer was rinsed briefly with 5ml BSS then serum free medium was added and flasks incubated for 24 hours. The

medium was removed, the monolayer rinsed as before and incubated in 5ml DMEM containing 100uCi [^{3}H]fucose (specific activity 70Ci/mmol) or [^{3}H]galactose (specific activity 31Ci/mmol) for 48 hours. After incubation with the radiolabel the medium was removed, centrifuged 2500xg for 5 minutes. The supernatant was removed and the protease inhibitor PMSF was added to give a final concentration of 1mmol/l and frozen at -20°C until ready for concentration (section 5.4.2.). The cells were scraped off using a rubber policeman, rinsed in 5ml DMEM and centrifuged 400xg 5 minutes. The pellet was resuspended in 1ml DMEM and frozen at -70°C.

5.4.2. SDS-PAGE and fluorography.

The medium containing radiolabelled glycoproteins released from cell culture (section 5.4.1.) was thawed and simultaneously concentrated and desalted using centrifugal concentrators, (section 2.3.1.) to yield a final volume of about 300ul. Total protein determination, electrophoresis and fluorography was done as previously described in chapter 2.

5.5. Purification of Cell Line Glycoproteins.

5.5.1. Cell culture.

Cell lines from "working stocks" were grown to confluence in ten 80cm^{2} flasks (approx. 9x10^{7} cells)

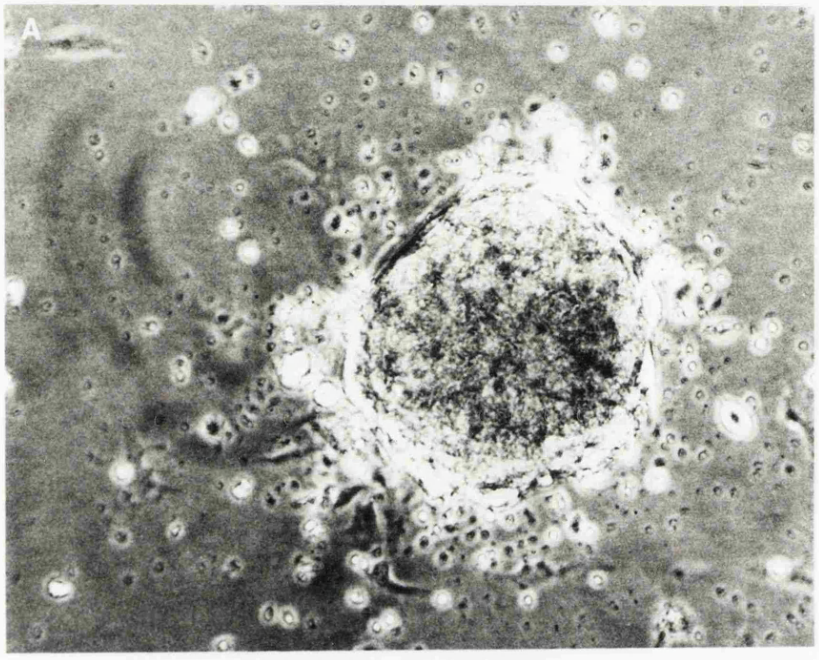
using the methods previously outlined in sections 5.3 and 5.4. The medium was removed, centrifuged at 2500xg for 5 minutes and the supernatants pooled. PMSF was added to give a final concentration of 1mmol/l. The medium was then frozen at -20°C until required for concentration (section 5.5.2.).

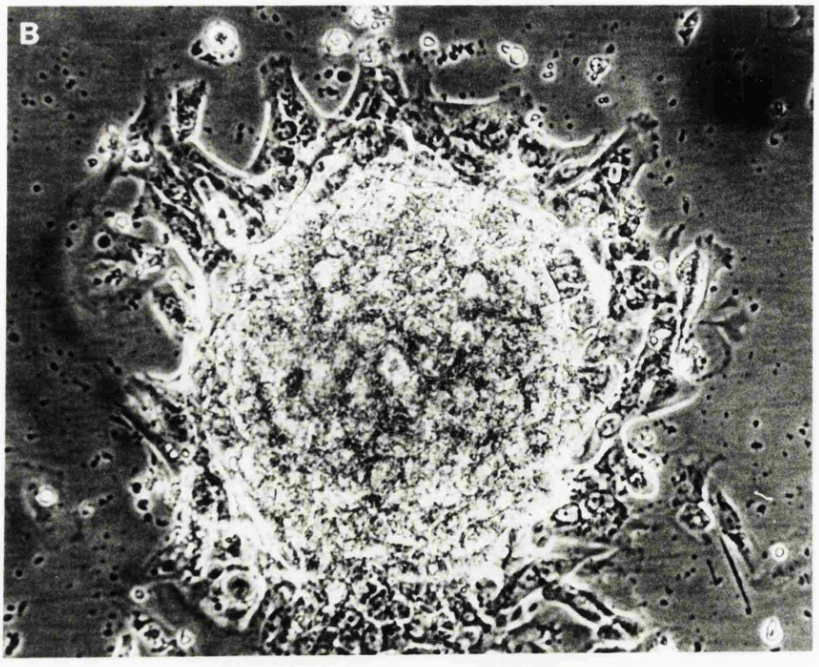
An alternative method of large scale cell growth was investigated briefly. The technique involved the growth of cells on cytodex beads in a commercial spinner culture system. This technique is able to provide cell growth at the very least ten times greater than conventional small scale cell culture. However it was soon found that a considerable amount of time was required to empirically optimize the culture conditions for each cell line. Due to constraints of time this technique was not pursued.

5.5.2. Concentration and purification by SDS-PAGE.

The medium from the cell culture was concentrated as before (section 5.4.2.), to a final volume of about 1ml. The sample was electrophoresed as described in section 4.1.1. and the relevant bands excised. The minced acrylamide strips containing the separated glycoprotiens were placed into individual dialysis bags (pre-treated as described in section 2.3.1.) containing the gel pieces and 10ml SDS-PAGE resolving gel buffer (section 2.3.3.). The sealed dialysis bags were placed in a horizontal gel tank containing the SDS-PAGE reservoir buffer (section 2.3.3) and electroeluted in a 4°C cold room overnight

Figure 5.1. Cells derived from dissaggregation (see text) of a grade III infiltrating ductal carcinoma.
Cell "clump" after dissaggregation (A); after 21 hours culture (B); after 94 hours culture (C).





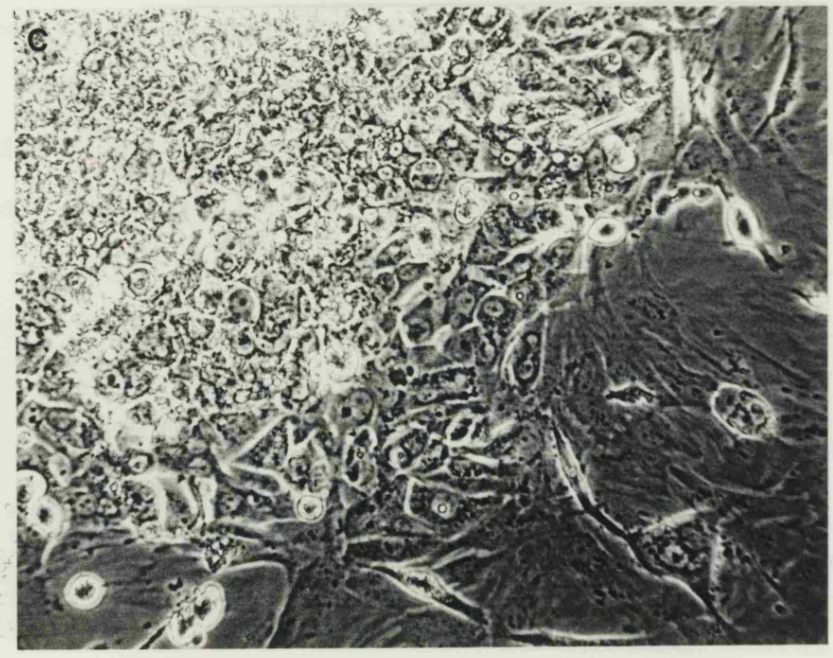


Figure 5.2. Different colony from same carcinoma (see figure 5.1.) after 94 hours culture.

at 20mA constant current. Before removing the dialysis bags from the buffer tank the current polarity was reversed for 1 minute. The contents of each dialysis bag was filtered through a polyallomer wool plug in a 10ml syringe to remove acrylamide pieces. The gel pieces were washed twice with 5ml of ultra-pure water and the filtrate was dialyzed first against 5l of 0.002% SDS in 0.1M NH_4HCO_3 then 7 hours against 5l of 0.1M NH_4HCO_3 at room temperature. The contents of each dialysis bag was lyophilized and resuspended in 1ml of 0.1M NH_4HCO_3. Two aliquots of each isolated glycoprotein was removed and subjected to SDS-PAGE, 7.5% single concentration gels. One sample was further processed for fluorography to assess purity, the other sample was silver stained (section 2.3.8.) using known amounts of BSA to estimate quantitative recovery of each sample.

5.6. Results.

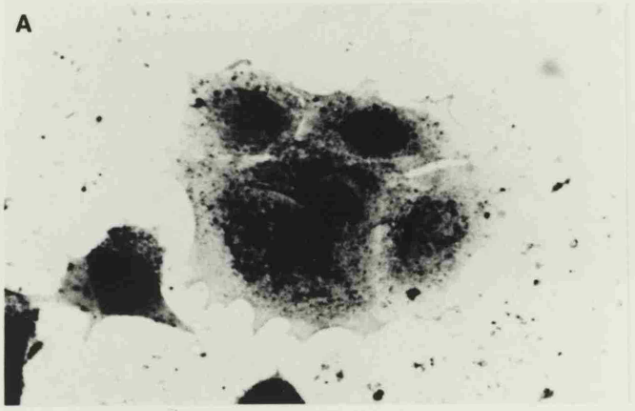
5.6.1. Primary tissue culture.

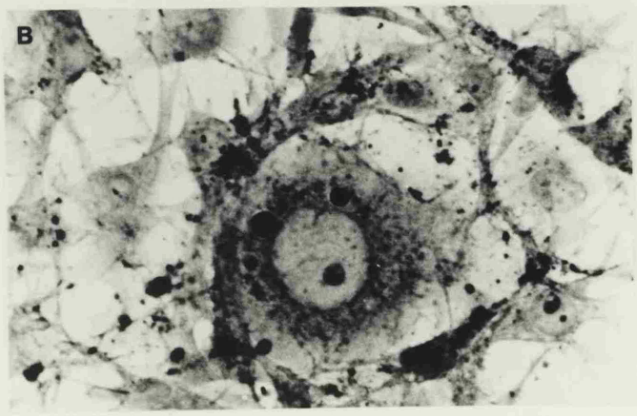
The limited success obtained with these techniques is shown in the time lapse photography of figures 5.1a-c. Many fibroblast-like contaminants were present around some colonies, as indicated by cytoskeletal stress fibres (figure 5.2).

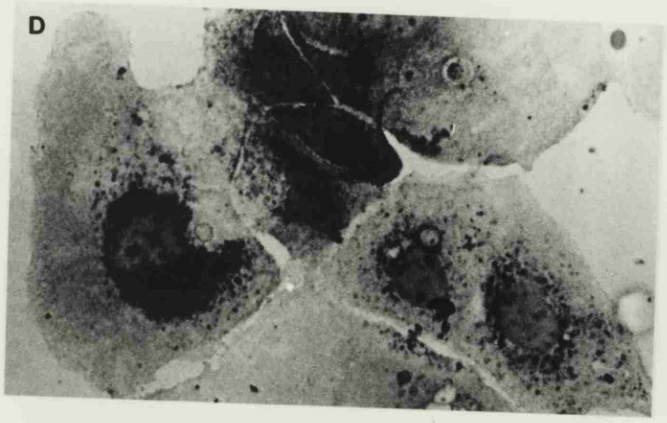
5.6.2. Immunohistochemistry.

Cell preparations using the Labtek slides were

Figure 5.3. Cell line preparations on Labtek slides showing reactivity of the polyclonal antisera P-5252; MCF7 (A); HS578T (B); T47D (C); BT20 (D and E); MDA MB 231 (F); MDA MB 468 (G); HBL100 (H); and BT474 (I). ICL=intracytoplasmic lumen.



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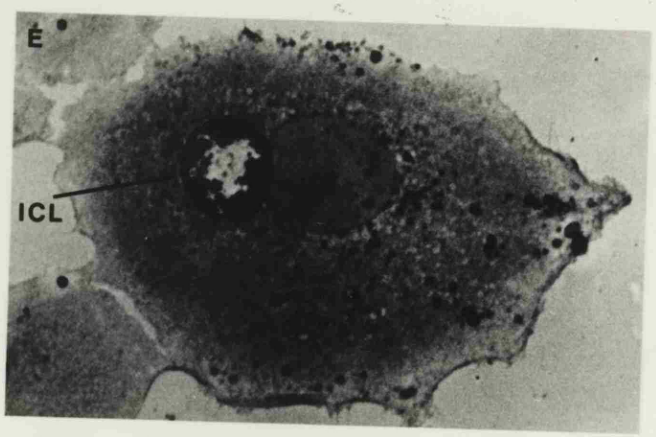
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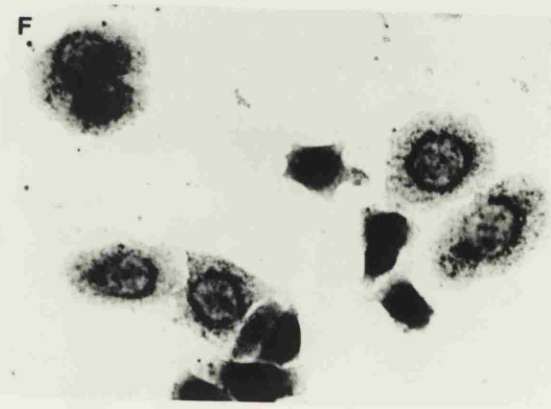
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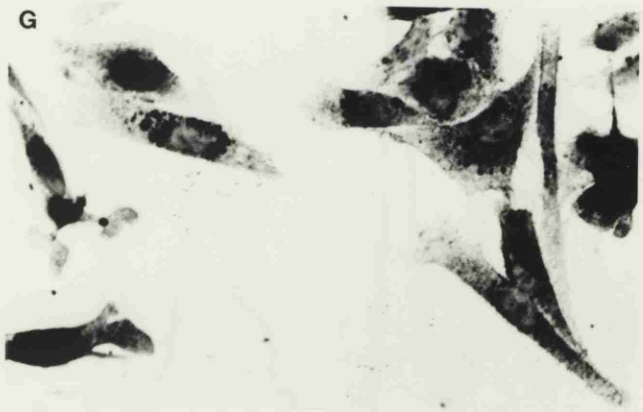
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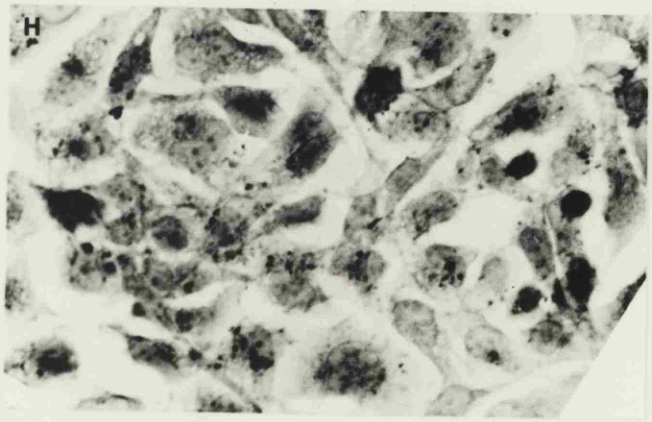
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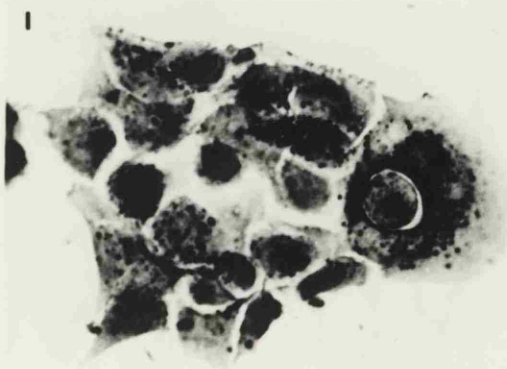
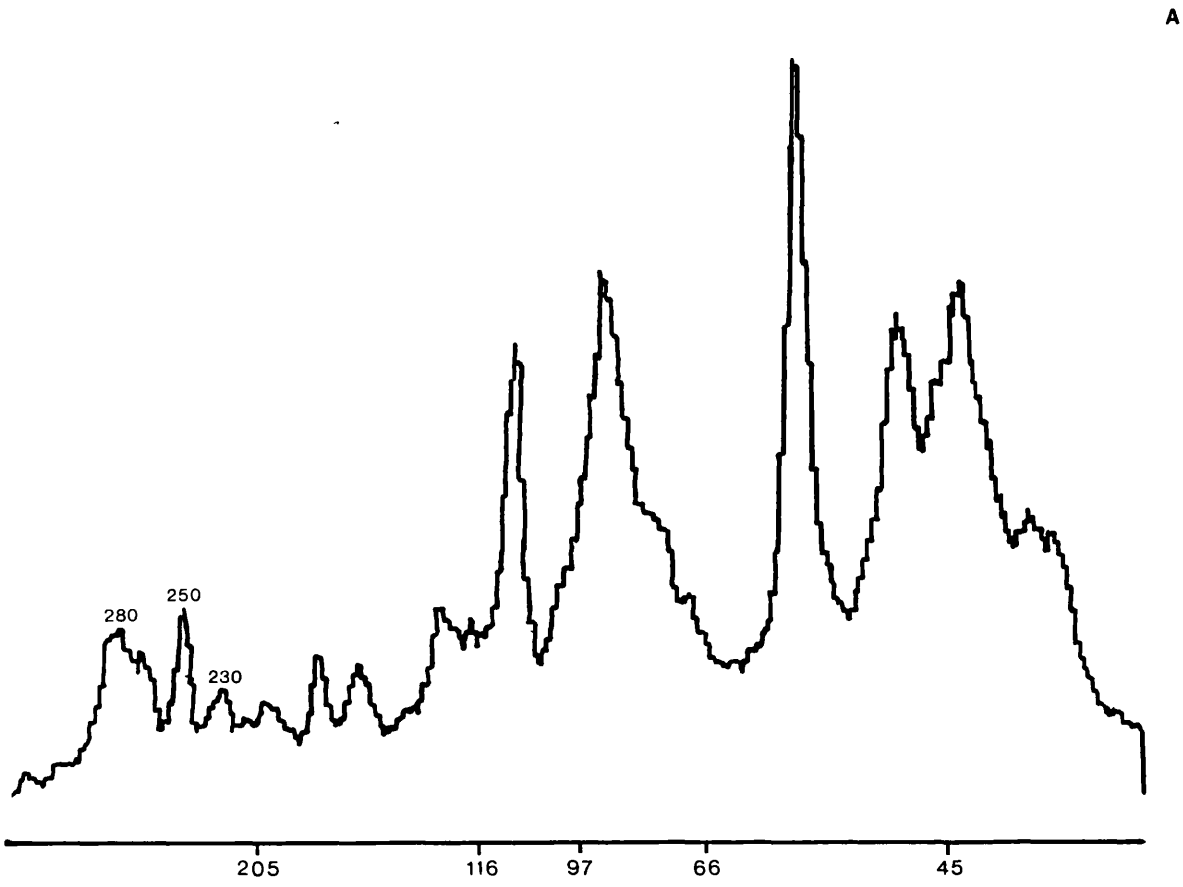
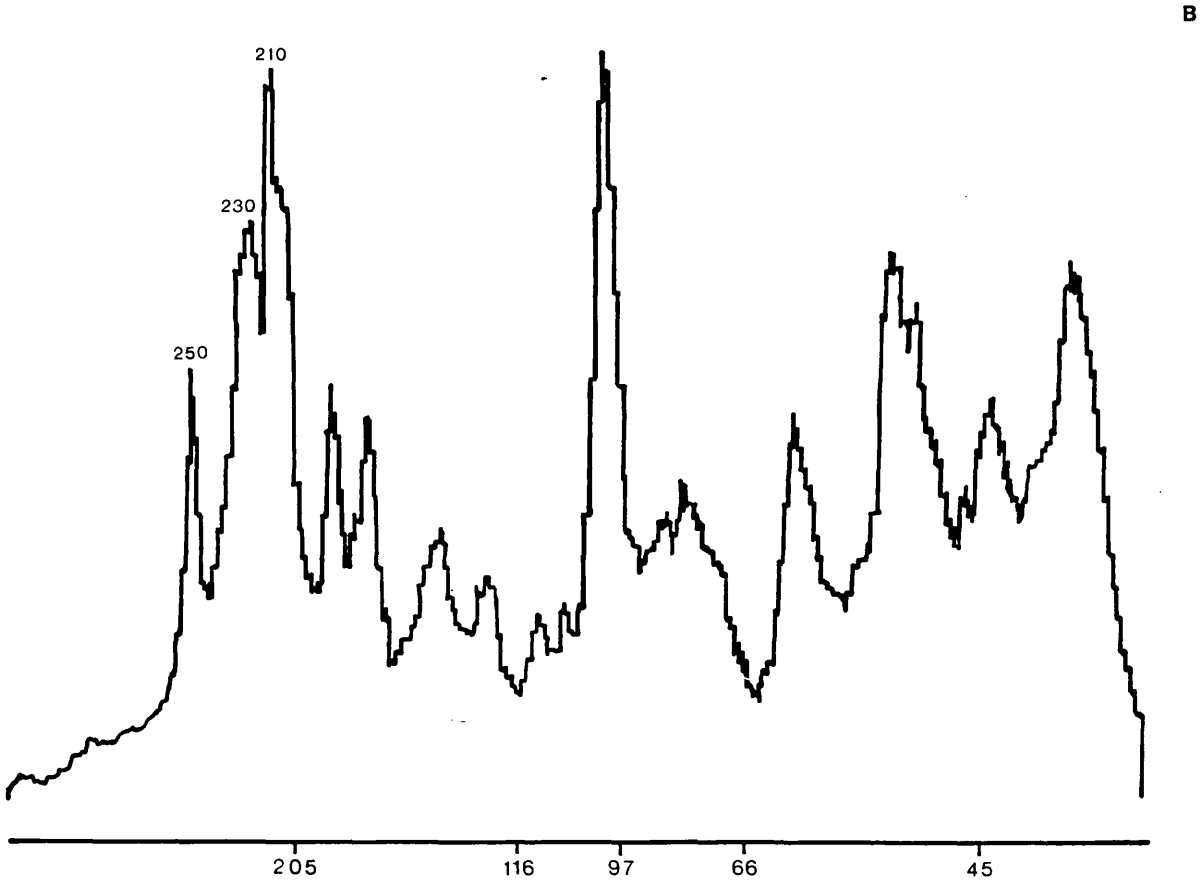

I

Figure 5.4. Densitometry tracings of SDS-PAGE showing fucosylated glygoproteins released from cell lines during culture; MCF7, A; HS578T, B; T47D, C; BT20, D; MDA MB 468, E; MDA MB 231, F.





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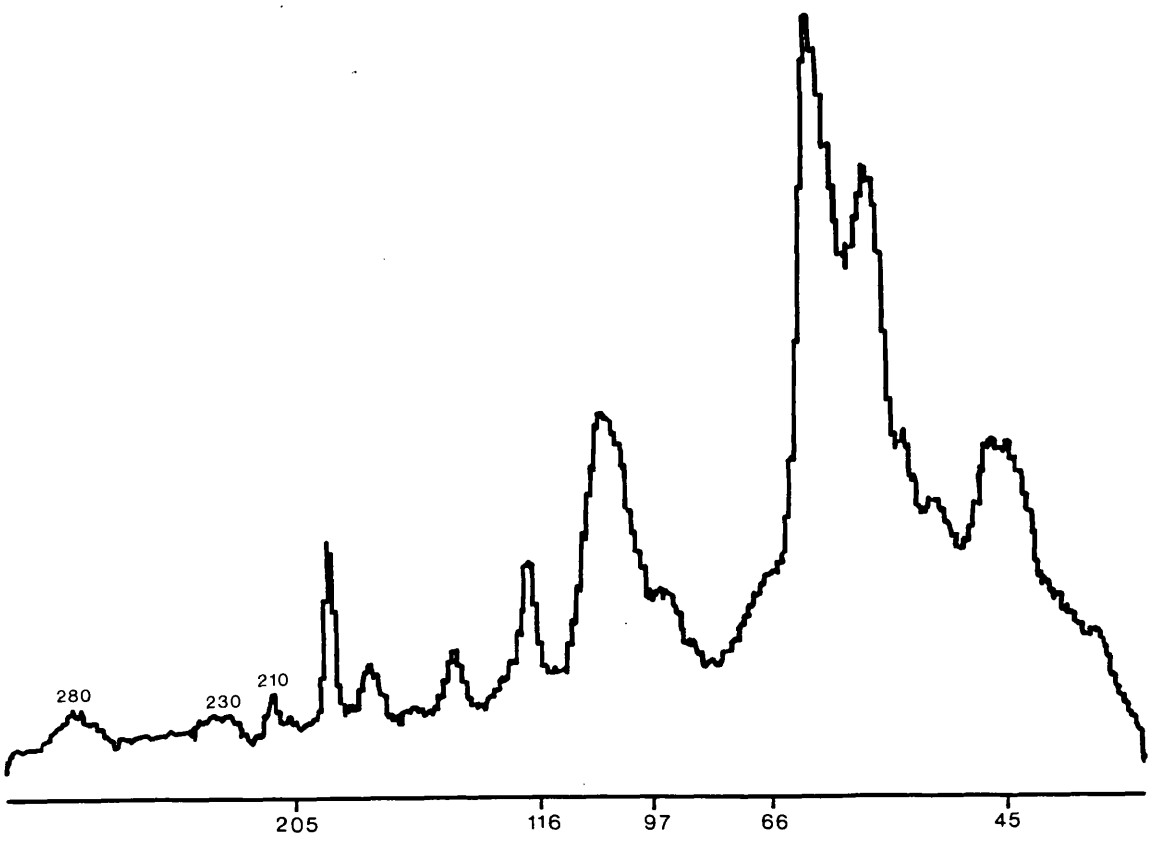

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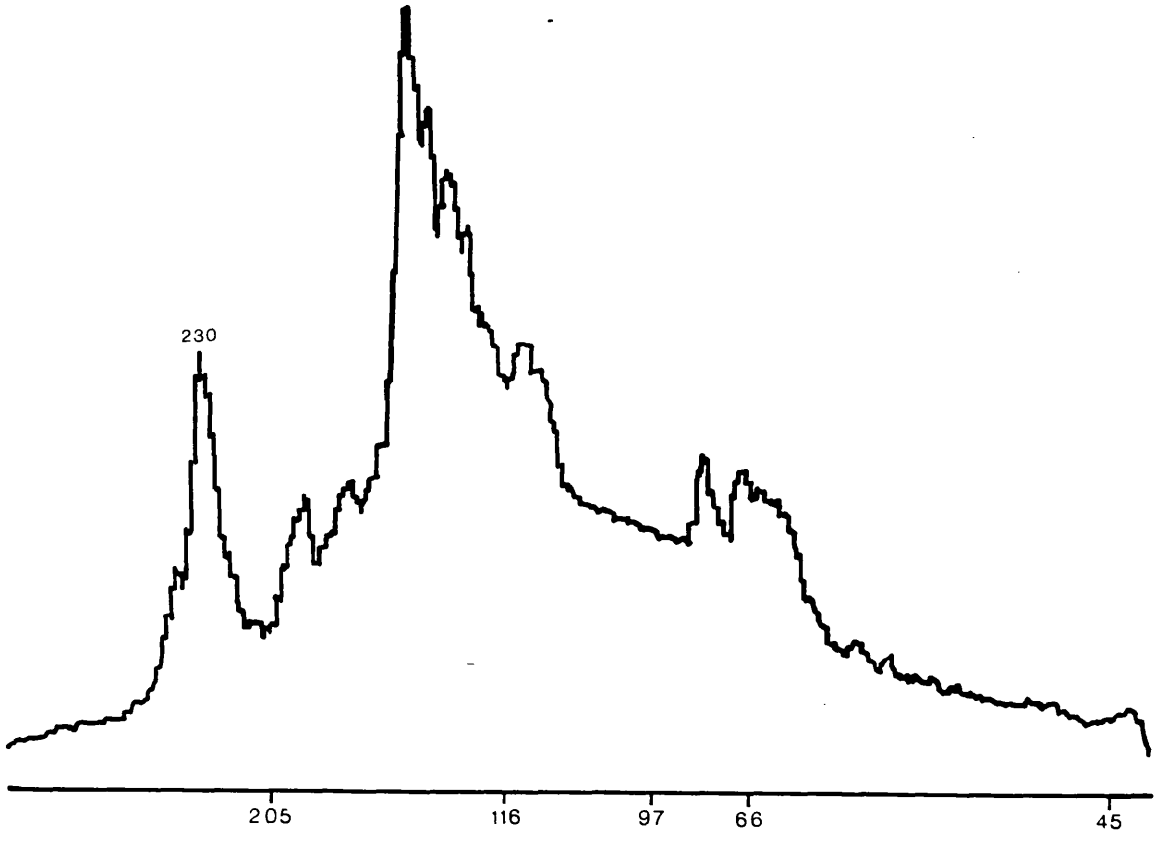

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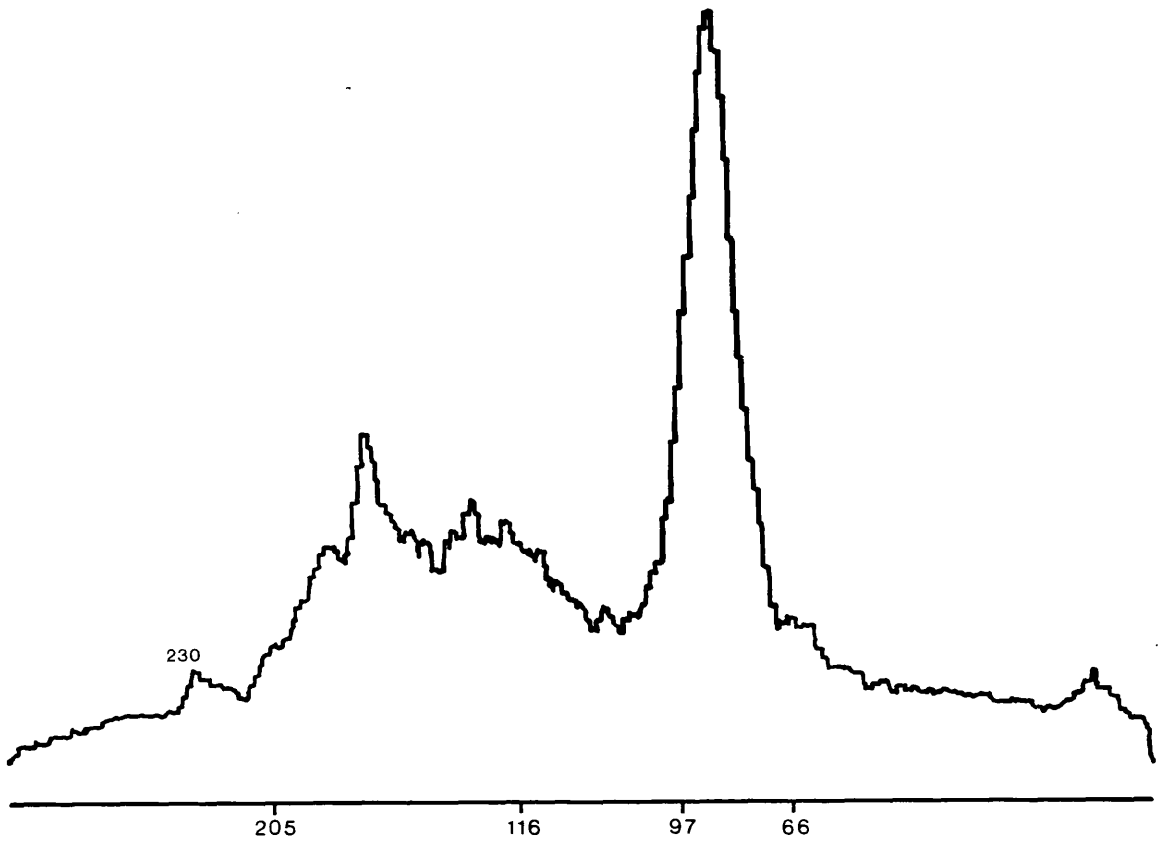

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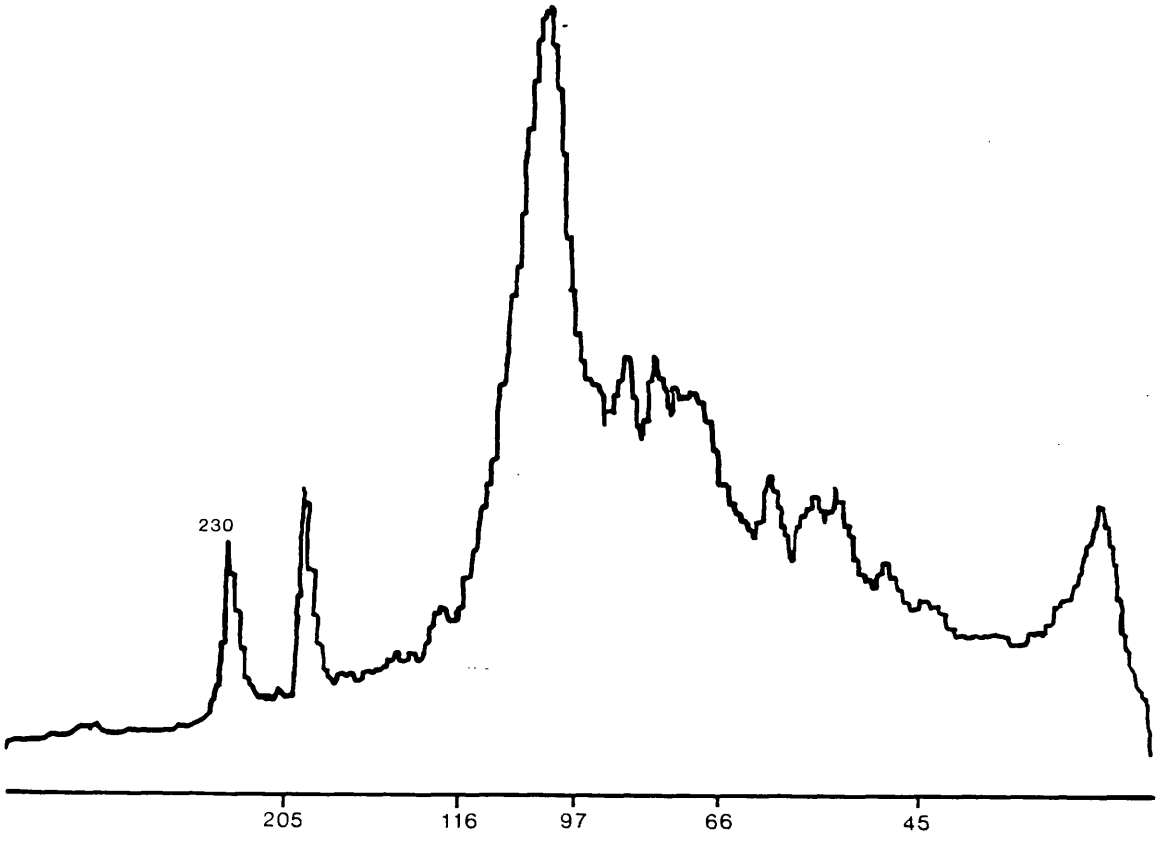


Table 5.1. High molecular weight glycoprotein expression in cell lines. F = fucose, G = galactose.

CELL LINE	ESTROGEN RECEPTOR STATUS	ORIGIN	GLYCOPROTEIN EXPRESSION (kD)			
			210	230	250	280
MCF7	POS	METASTASIS	+G	+FG	+FG	+FG
HS578T	NEG	PRIMARY	+F	+F	+FG	+G
T47D	POS	METASTASIS	+/-F	+/-F	-	+FG
BT20	NEG	PRIMARY	+G	+F	-	+G
MDA MB231	NEG	METASTASIS	+G	+F	+/-G	-
MDA MB468	NEG	METASTASIS	-	+/-F	-	-

more informative as to the staining patterns in the cell lines. The reactivity of the antisera P-5252 was focal and cytoplasmic (figure 5.3.a-5.3.i), with the MCF-7, BT20 and MDA MB 468 cell lines showing focal juxta-nuclear staining. The intensity of staining was variable, with the HBL100 line showing the weakest staining.

5.6.3. Medium analysis.

The glycosylation patterns of the breast cell lines exhibited marked variability between the individual cell lines as well as many similarities, in particular the high molecular weight glycoprotein of 230kD (table 5.1). This glycoprotein was identified in all six cell lines tested and labelled predominantly with [^{3}H]fucose (figure 5.4). Incorporation of [^{3}H]galactose in the group of high molecular weight glycoproteins was similar to that observed for fucose, with the exception of the glycoprotein of 230kD (table 5.1). Quantitative differences between the cell lines were apparent in the expression of the glycoprotein of 230kD. The expression of this glycoprotein was greatest in the two cell lines, HS578T and BT20, which were derived from primary carcinomas. There did not appear to be a relationship between glycosylation or glycoprotein expression and estrogen receptor status or origin of the cell line (table 5.1). There was considerable heterogeneity between the cell lines with respect to the number of glycoproteins incorporating the radiolabel, with the HS578T cell line showing the

highest number.

The immunohistochemical reactivity of P5252 coincides with the cell line expression of the fucosylated glycoprotein of 230kD in the six cell lines tested (table 5.1).

5.6.4. Purification of glycoproteins.

The protocol used for isolating the glycoproteins expressed in media from cell line cultures failed to yield sufficient quantities. Despite the presence of a whitish lyophilate at the end of the concentration procedure, prior to electrophoresis, no silver stained material was detected, and this was later deemed to be SDS.

5.7. Discussion.

The preliminary attempts at primary culture highlighted the complexity and "fine-tuning" required to determine the optimum culture medium that would select only for epithelial cell growth. Due to the presence of the more rapidly growing fibroblasts, the epithelial-like colonies were quickly overgrown and subsequently difficult to isolate. The contamination of epithelial tumour cells by rapidly proliferating fibroblasts is a recognized problem in primary culture of the breast (Whitescarver, 1974; Whitehead, 1976; Burg, *et al.*, 1989). The development of more defined selective medium and micromanipulation techniques

might prove to be more successful, although the isolation of cells at low densities is known to have a detrimental effect on cell growth due to the reduced amount of autocrine growth factors in the surrounding medium. Future work in this area could involve co-culture techniques where the primary epithelial cells would be cultured in tandem with established cell lines, which may produce factors that would enhance the growth of any malignant cells present. This co-culture method would then avoid the problems of seeding or cloning cells at low densities. However, recent advances in the knowledge of growth requirements of cells in culture (Ham & McKeehan 1979), will in time remove the serendipitous aspect of primary cell culture.

The reactivity of the antisera P5252 paralleled the expression of the glycoprotein of 230kD in those cell lines tested, showing some small quantitative differences; the different staining intensity seen in the cell lines could be the result of cells in different phases of growth. These differences could also be attributed to the structural homology previously identified between the glycoproteins of 230 and 250 kD and the specificity of the antiserum as discussed in chapter 4. The localization of staining showed similarities to the malignant primary tissue tested (see chapter 4), with the BT20 cell line showing marked focal staining of intracytoplasmic lumina or ICL's. The staining around the nucleus seen in three of the cell lines could be associated with

the ER or Golgi complex. The concentration of reactivity in those areas, to the general exclusion of membrane or diffuse cytoplasmic staining, implicates a possible defect in the transport of these antigens. The greater expression of the glycoprotein of 230kD in HS578T and BT20 correlated with the origin of these cell lines; both were obtained from primary tumour tissue of an elderly patient presenting with adenocarcinoma of the breast. This correlation may be fortuitous in respect of the small number of cell lines available, or it may indicate a real correlation between the expression of the glycoprotein in primary tissues and its reduced expression in metastases. However, a detailed comparison of primary tumours and their metastases would be required to validate this relationship.

The immunohistochemical and biochemical similarities between the cell lines and the primary tissues supports the use of the cell lines as a model system for the study of these high molecular weight glycoproteins.

The method of purification of the glycoproteins from cell lines resulted in considerable losses. Using the techniques described these losses may have been acceptable had the volume of starting material been much higher. The fixing and staining procedures (used to aid molecular weight estimation and to ensure that adequate separation had occurred), although short, may have prevented the efficient elution of the high molecular weight glycoproteins from the gel matrix,

despite the lengthy electroelution procedures. Furthermore, it is likely that the use of dialysis tubing, although treated as mentioned in chapter 2, may have increased these losses through adsorption of the glycoproteins to the tubing surface. If this method was repeated the use of a low molecular weight blocking agent, acting in a similar fashion to that used in Western blotting or immunohistochemical techniques, may reduce the losses due to adsorption. Residual blocking agent remaining in the sample could then be removed by ultra filtration. The final precipitate, present after lyophilization, upon inspection and solubilization appeared to be SDS which was not removed by dialysis, thus highlighting that the dialysis procedure was not extensive enough and more frequent changes of buffer would be necessary in future.

Attempts to isolate the high molecular weight glycoproteins for further study may in future use the antiserum P5252 in an affinity chromatography column as a preliminary stage in a purification protocol.

CHAPTER 6

ANALYSIS OF PLASMA MEMBRANE FROM BREAST TISSUE.

The high molecular weight glycoproteins, described in the preceeding chapters could be derived via two pathways; active secretion (outlined in chapter 1, section 1.1.4.) or membrane protein shedding/turnover. In the latter pathway, release of glycoproteins from the plasma membrane would arise from proteolytic degredation. In the tumour cell the increase of catabolic enzymes (see chapter 1, section 1.2.2.), and overall cell turnover could contribute to the elevated expression of certain membrane components released into the extracellular environment.

In order to define more closely the role of this group of glycoproteins it was neccesary to investigate their cellular localization biochemically, and complement the earlier findings from the immunohistochemistry (chapter 4).

This chapter describes the preliminary studies involved in the analysis of plasma membranes derived from both primary tissue and breast cell lines. Work

initially concentrated on obtaining plasma membranes from organ cultures of primary breast tissue using mechanical disaggregation and density gradient centrifugation techniques. However, considerable difficulties were experienced in this approach due to the small quantities of tissue available and the high stromal content of the tissue, although the latter varied considerably between tumours. It was therefore decided to use those breast cell lines discussed in chapter 5, as a model system to obtain sufficient numbers of epithelial cells (free from stromal contamination) from which plasma membranes were prepared. A mechanical homogenization procedure was used as before, but with a different type of separation method which is dependant on the chemical composition and behaviour of membrane fragments in a two-phase polymer system (Brunette & Till, 1971). It was hoped that this latter separation technique would provide better membrane preparations. This technique and others used in the preparation of membranes described in this chapter are later discussed as to their suitability for this study. The constraints of time precluded a detailed analysis of the membranes prepared by these methods.

Another approach done in parallel with the membrane preparations was to determine the localization of these glycoproteins by fractionating homogenates of breast tissue, on the basis of solubility in water (Dunbar, 1988). This method, whilst not specifically isolating particular groups of

organelles, can allow the fractionation of cells into two compartments; water soluble and water insoluble. The rapid and easy fractionation of tissue in this manner had many advantages over the more classical centrifugal fractionation techniques; (i) reduced exposure to proteolysis due to short processing time, (ii) no detailed analysis required to determine purity of preparation (e.g., electron microscopy and enzyme activty), and (iii) small quantities of tissue can be processed with minimal losses.

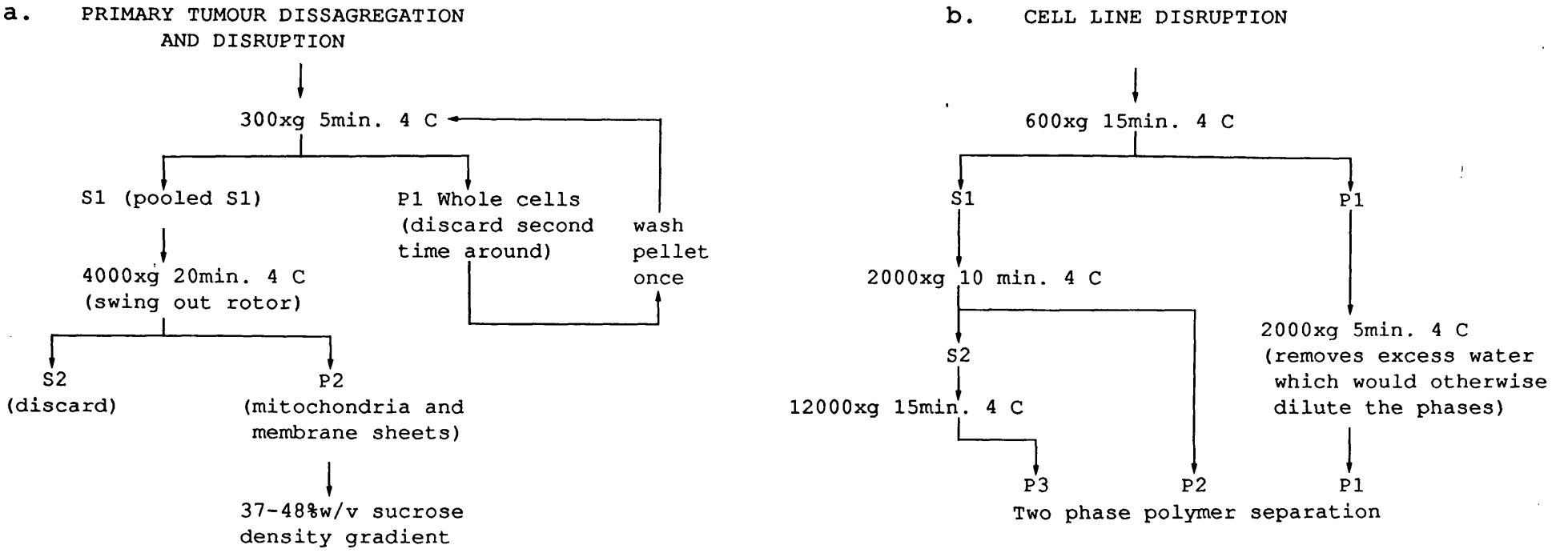
The methods and findings of the approaches to this area of work although preliminary, provide the basis for a future detailed study of the localization of glycoproteins in different cellular compartments.

6.1. Membrane Preparation From Primary Tumours.

6.1.1. Tissue disaggregation techniques.

Several mechanical methods of disrupting breast tumour tissue were tried with limited success.

Figure 6.1. Differential centrifugation protocols for preparation of membrane fractions from primary tumours (a) and cell lines (b).



Sonication. Breast tissue from organ culture was placed in a corex tube with 5ml of ice cold 5mM Tris buffer containing 0.25M sucrose and 0.5mM PMSF. The sample, cooled on ice, was sonicated (Soniprep 150) in short bursts for a total time of 5 minutes using a 3mm tip at 14u amplitude. This treatment alone had little effect on the gross morphological appearance of the tissue blocks.

Mechanical homogenization. Three types of homogenizers were tried; a loose fitting (0.6mm) motorized Potter-Elvejhem apparatus, a loose fitting Dounce homogenizer and a rotary homogenizer with stainless steel blades. Both the Potter-Elvejhem and the Dounce homogenizers failed to disrupt even the gross structure of the tissue blocks, and only with a combined enzyme technique would this type of apparatus homogenized the tissue. Of the three pieces of apparatus, the rotary homogenizer was found to be the most effective. Tissue blocks were placed in a corex tube containing 10 ml of the hypotonic buffer, 10mM Tris HCl (pH 8.0) with 0.5mM PMSF at 4°C for 15 minutes. The tissue was homogenized on ice with one 10 second burst at high speed and 0.2M CaCl was immediately added to stabilize the membranes. The homogenate was then ready for the fractionation of the plasma membrane fragments.

6.1.2. Cell fractionation and membrane purification.

The tissue homogenate was subjected to differential centrifugation as shown in figure 6.1a.

and the final pellet (P_2) comprising mitochondria and membrane sheets, was resuspended in 2ml of buffer containing 5mM Tris HCl (pH 8.0), 0.25M sucrose, 0.5mM PMSF and 0.5mM $CaCl_2$. To this sample 15ml of 60% (w/w) sucrose solution was added and the final refractive index of the solution adjusted to give a 48% (w/w) sucrose solution. To a thin walled polyallomer centrifuge tube containing 15ml of 37.2% (w/w) sucrose solution, 15ml of the adjusted 48% (w/w) sucrose solution was added. This was done by carefully under-layering the less dense solution using a peristaltic pump on its slowest speed setting. This 2-phase sucrose system was then overlayed with 2ml of buffer and centrifuged at 150000xg for 4 hours at 4°C. The diffuse white band in the 37% region was aspirated and diluted with three volumes of buffer containing 5mM Tris HCl (pH 8.0) containing 0.5mM PMSF and 0.5mM $CaCl_2$. The membranes were finally harvested by centrifuging at 100000xg for 40 minutes at 4°C and processed for enzyme assay and electron microscopy.

6.1.3. Membrane processing for electron microscopy.

The membrane pellets were disrupted and fixed in 4% paraformaldehyde 5% gluteraldehyde after Karnovsky, (1965) (appendix B) at 4°C for 1 hour. Between subsequent washing staining and dehydration steps the membrane pellets were recovered by centrifuging in a microfuge at about 12000xg for 20 minutes. The pellets were then washed in two changes of cacodylate buffer (appendix B) for 10 minutes each

and further fixed in 1% Osmium tetroxide in cacodylate buffer at 4°C for 1 hour. After two 10 minute washes in cacodylate buffer the pellets were stained in freshly prepared 1% uranyl acetate in ultra-pure water for 45 minutes. The pellets were dehydrated through 50%, 70% and 90% alcohols to absolute ethanol (10 minutes each) transfered to propylene oxide in ethanol (1:1) for 10 minutes then two changes of fresh propylene oxide also for 10 minutes. The membranes were transfered to Emix resin in propylene oxide (1:1) and incubated at 37°C for 30 minutes. The samples were placed in neat resin at 37°C for 1 hour and finally placed in capsules with fresh resin and allowed to polymerize at 60°C overnight.

6.1.4. 5′ Nucleotidase assay.

The radioassay of Avruch and Wallach (1971), was used to detect the presence of the ecto-enzyme 5′ nucleotidase (5′NT) and hence estimate the purity of plasma membrane preparations. The reaction mixture comprised, 250ul of 100mM Tris HCl (pH7.4) containing 40uCi [2-^{3}H]adenosine 5′-monophosphate ammonium salt (specific activity 20Ci/mmol) and 250ul of freshly prepared unlabelled 400uM AMP. The reaction was initiated by the addition of 10ul of membrane preparation or diluted 5′NT for calibration standards, and incubated for 30 minutes at 37°C. The reaction was terminated by addition of 100ul 0.15M $ZnSO_4$. Unreacted AMP and other proteins were precipitated with 100ul 0.15M $Ba(OH)_4$. After leaving the stopped reaction at

4°C for 30 minutes with occasional agitation, the samples were then centrifuged at 12000xg for 10 minutes and 250ul of the supernatant added to 5ml of the toluene/Triton X-100 (7:3) scintillation cocktail, containing 0.5% 2,5-diphenyloxazole (PPO). In order to reduce interference from chemiluminesence, samples were left at room temperature for 24 hours before counting. Before samples were run a brief interfacing experiment was done (Appendix F), to ensure the ratio of scintillant/aqueous sample volume did not result in phase separation of the scintillation fluid.

6.2. Fractionation of Tumour Homogenates by Solubility.

6.2.1. Tissue disaggregation and solubilization.

Radiolabelled frozen (-70°C) tissue from organ culture (200mg wet weight-duplicate samples from the same carcinoma were pooled) was pulverized in a stainless steel mortar and pestle (designed by the author and made by the University workshop). The fragmented tissue was rapidly thawed, added to ultra-pure water containing 0.5mM PMSF and mixed well for 1 minute. After leaving to stand at 4°C for 15 minutes the samples were again mixed for 1 minute. The samples were then processed for fractionation.

This method of tissue disaggregation although proved technically more problematic, yielded a similar homogenate to that produced by the rotary homogenizer.

6.2.2. Fractionation of water soluble and insoluble fractions.

Tissue homogenates prepared as described in section 6.2.1. were centrifuged in pre-weighed tubes at 100000xg for 1 hour using a swing out rotor precooled to 4°C. The supernatant (S_1) was decanted, taking care to avoid including the surface fat layer, and placed at 4°C. The pellet (P_1) was resuspended in ultra-pure water containing 0.5mM PMSF mixed for 1 minute and re-centrifuged. The supernatants from both centrifugation steps were pooled; water soluble fraction (WSF). The washed pellet (P_1) was drained to remove excess water; water insoluble fraction (WIF). All samples were subsequently lyophilized and re-weighed before solubilizing for 2 hours in 2ml SDS sample buffer (section 2.3.) at room temperature. Solubilizates were centrifuged at 12000xg for 10 minutes to remove insoluble matter and the supernatants electrophoresed by SDS-PAGE.

6.2.3. SDS-PAGE, fluorography and silver staining.

Polyacrylamide gel electrophoresis on 7.5% gels was performed as described in section 2.3. A number of unlabelled samples were processed for SDS-PAGE and then silver stained according to section 2.3.8. Using this one dimensional approach there were too many silver stained proteins to effect adequate resolution of individual bands (section 6.4.). Nevertheless the techniques were repeated with the organ culture radiolabelled samples since only a proportion of the

silver stained bands would be visualized by fluorography (section 2.3.9.).

6.3. Membrane Preparation From Cell Lines.

6.3.1. Cell disruption technique.

Confluent cell monolayers were removed using 4mM EDTA washed, pelleted at 300xg 5 minutes and resuspended in a hypotonic buffer of 30mM $NaHCO_3$ (pH 7.0) containing 0.5mM PMSF, giving a cell suspension of $2.0x10^7$ cells/ml. The cell suspension was incubated at 20°C for 15 minutes to allow the cells to swell before cooling to 4°C and subjecting the suspension to the liquid sheer forces of a tight fitting (0.07mm clearance, teflon plunger) Dounce homogenizer. The number of strokes required to disrupt the cell suspension (dependant on the cell line, but were found to be between 15 and 30) was carefully monitored by phase contrast microscopy. The resultant cell homogenate was then subjected to differential centrifugation as described below.

6.3.2. Cell fractionation and membrane purification.

Cell homogenates were fractionated by differential centrifugation as outlined in figure 6.1b. after Gruber, et al., (1984), before purification on an aqueous 2-phase system. The aqueous 2-phase polymer separation system used was that of Brunette & Till (1971). The 2-phase system was

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prepared by adding the following solutions in a separating funnel and allowing to separate at 4°C; 200g of 20% (w/w) dextran 500 in ultra-pure water; 103g of 30% polyethylene glycol (PEG) in ultra-pure water; 99ml of ultra-pure water; 333ml of 0.22M phosphate buffer (pH 6.5) and 80ml of 10^{-2}M ZnCl. When the two phases had settled, as defined by a sharp refractile difference between the two layers, the phases were collected separately. The pellets P1, P2 and P3 from the differential centrifugation were pooled, resuspended in 10ml of top phase, and 10ml of the bottom phase was added. The two phases were mixed and centrifuged at 12000xg for 10 minutes, the supernatant containing the membranes and the two phases was removed, mixed and re-centrifuged to remove any trapped contaminants. The membranes at the interface were collected and diluted in 4 volumes 30mM $NaHCO_3$ and centrifuged at 12000xg for 20 minutes. The supernatant was discarded leaving the last 1ml undisturbed, and the pellet was resuspended in this and stored at 4.C until required for assay or microscopy.

6.4. Results.

6.4.1. Plasma membranes from primary tissue.

Preparation of the plasma membrane component from primary tissue was beset with difficulties. Sonication of 1-2mm^3 tissue blocks was insufficient to

Figure 6.2. Photoelectron micrographs showing plasma membrane preparations from primary tissue; benign A; carcinomas B and C. These three preparations were also tested for 5'NT activity, (see table 6.1.)

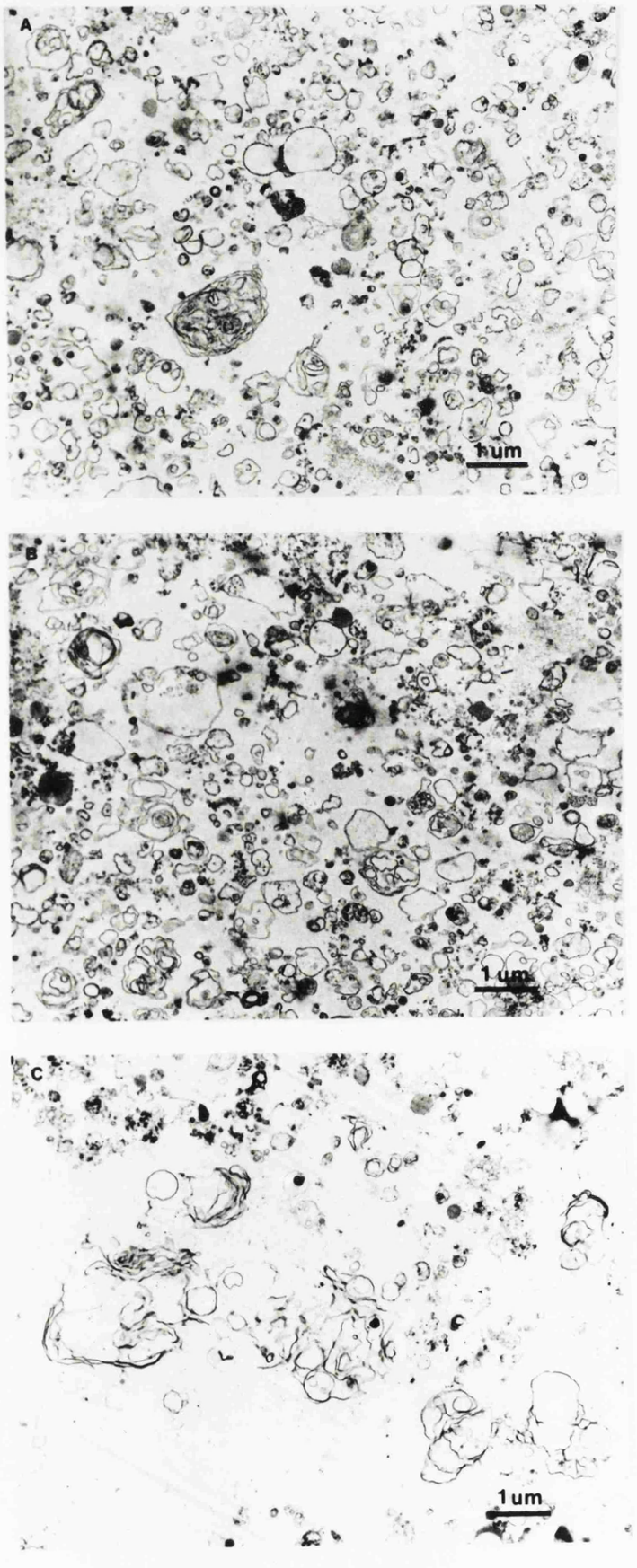


Table 6.1. Results of the 5'NT assay for the samples shown as photoelectron micrographs in figure 6.2.

MEAN CPM	CONTROL CORRECTED CPM	5'NT ACTIVITY (Units $x10^{-3}$)	
151,387	129,932	5.6	
63,542	42,087	2.8	
37,017	15,562	1.4	
33,394	11,939	0.7	
24,067	2,612	0.56	
21,455	0	no enzyme	
20,466	-	0.00	A
28,869	7,414	0.56-0.7	B
36,188	14,733	0.7-1.4	C

(counting efficiency 41%)

effect gross morphological change. The Potter-Elvejhem and Dounce homogenizers while causing some cell disruption on the external surfaces of the tissue blocks, failed to make a significant impression on the gross structure. The rotary homogenizer, although initially thought to be too harsh, produced a turbid tissue homogenate which subsequently yielded the membrane preparations shown in figure 6.2. This technique however still did not disrupt all the tissue blocks effectively. The success of these disaggregation techniques was dependant on the nature of the tumour, with very cellular tumours yielding the best results (figure 6.2.B). The size of membrane fragments seen in the electron micrographs varied considerably, from large sheets to small vesicles. In all three cases there was a large amount of unspecified debris.

The 5'nucleotidase assay performed on membrane samples from the same cases (table 6.1), did not show any correlation with the membranes observed by electron microscopy (figure 6.2). Indeed one sample showed no enzyme activity whatsoever.

6.4.2. Fractionation of tissue by solubility.

The fractionation by water solubility revealed a complex mixture of components on a silver stained one dimensional gel (figure 6.3a). The water soluble fraction revealed a number of high molecular weight components >180kD. The water insoluble fraction was less clear with heavy smearing above 200kD and some

Figure 6.3A. Silver stained cellular components released after fractionation by water solubility; water soluble fraction (A-D); water insoluble fraction (E-H).

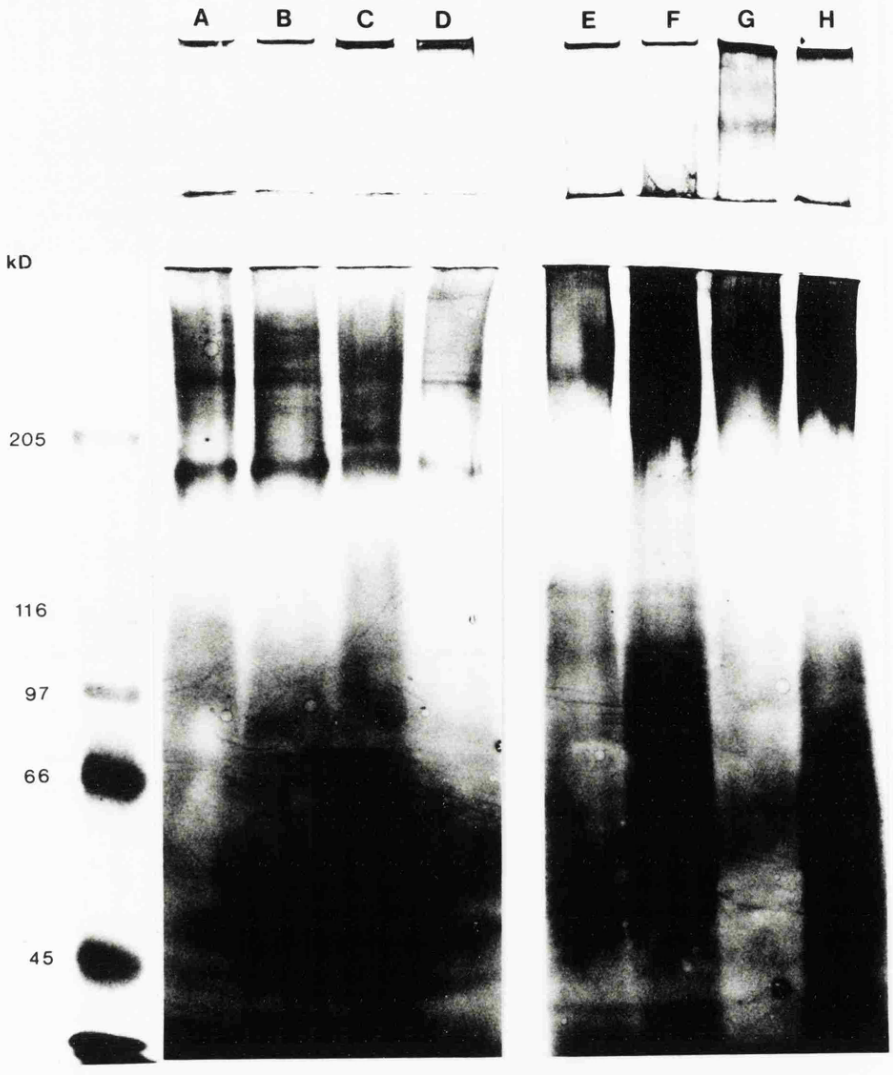


Figure 6.3B. Fluorograms showing fucosylated components released after fractionation by water solubility; water soluble fraction (A); water insoluble fraction (B).

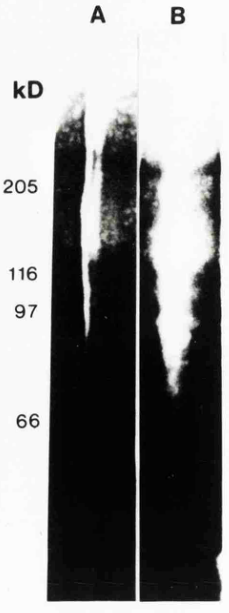


Figure 6.4. Phase contrast micrographs showing progressive cell disruption using a tight fitting Dounce homogenizer; cells before homogenization A; 10 strokes B; 20 strokes C; and 30 strokes D. (see text, section 6.3.1). Note appearance of "bare" nuclei (n) and other cell debris.

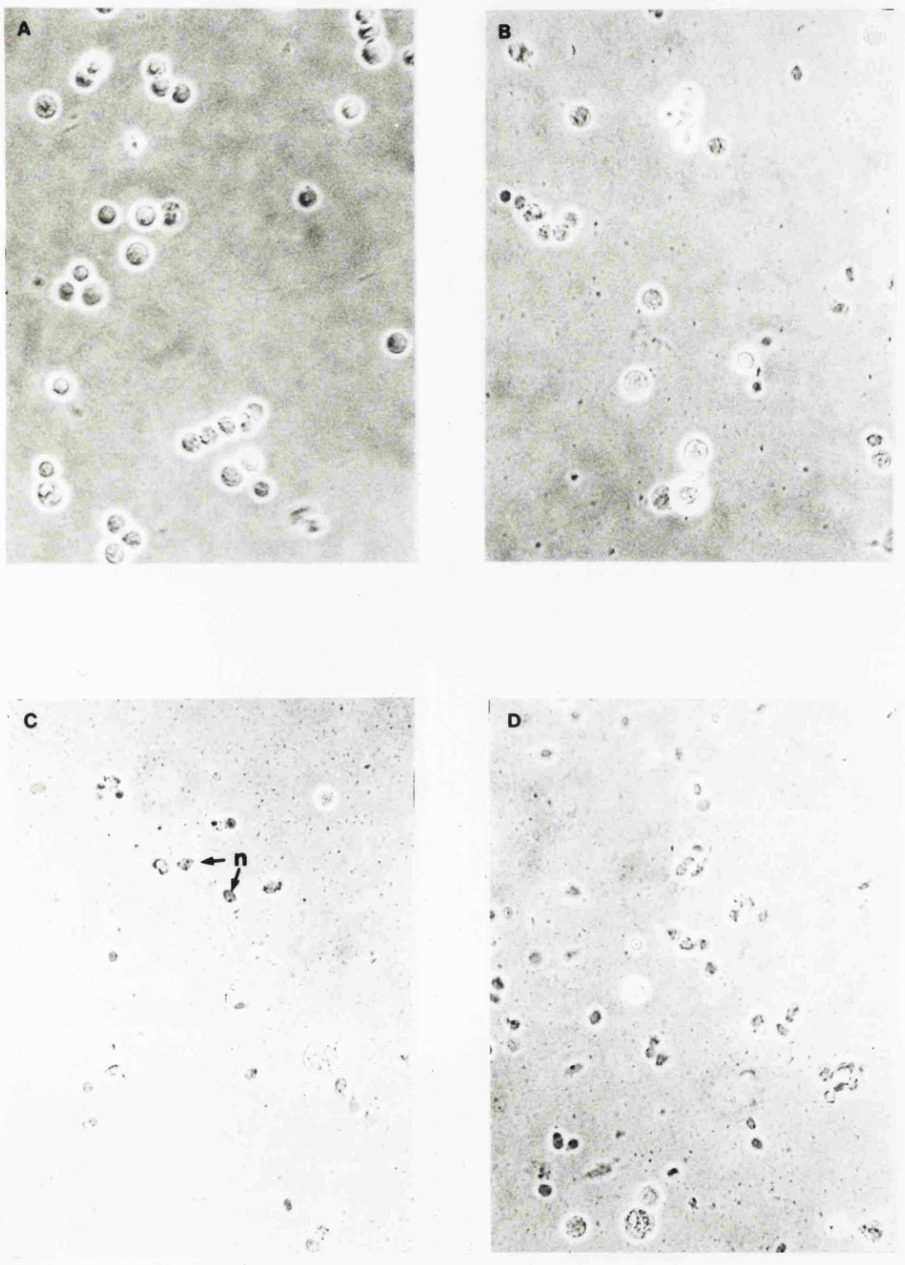
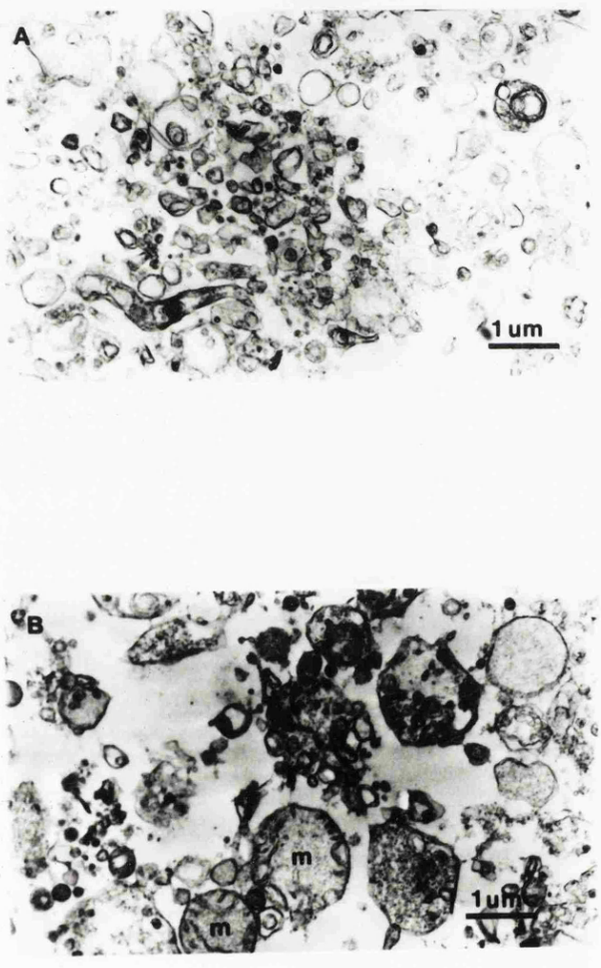


Figure 6.5. Photoelectron micrographs showing plasma membrane preparations from cell lines; MCF7 (A); and HS578T (B). Note large mitochondria-like structures (m).



material being retarded in the stacking gel (lane F and G). The lower section of the gel although not clear in the photograph showed a large number of bands present in both fractions. The fluorogram (figure 6.3b) shows the presence of a fucosylated glycoprotein at 230-250kD in both the water soluble and insoluble fractions. Further comparisons were obscured by smearing which shorter exposure times failed to clarify.

6.4.3. Plasma membranes from cell lines.

Disruption of the cell lines in suspension required only 15-30 strokes using a Dounce homogenizer. The phase contrast photomicrographs in figure 6.4. show this disruption of cell structure. The aqueous 2-phase polymer separation system described in section 6.3.2. was easier to prepare and use than the sucrose density gradient separation of membranes from primary tissue. The cell line membrane preparations as determined by electron microscopy alone, showed a wide variation in the size of membrane fragments (figure 6.5), ranging from small vesicles to large membrane sheets. There was also a small amount of particulate matter present which was not recognized as being of membrane origin.

6.5. Discussion.

The problem of isolating predominantly

epithelial cells from a heterogenous block of tissue is compounded by the tissue architecture: dense stromal tissue (section 1.3.) provides a near impenetrable barrier to mechanical disruption and subsequent membrane isolation. The mechanical "spill-out" method of Lasfargues and Ozzello (1958), has in the past been successful (Leibovtz, *et al.*, 1976; Burg, *et al.*, 1989), although this technique like many others is dependant on a high epithelia to stroma ratio, which varies widely between tumours. Enzymatic techniques, e.g. using collagenases, may succeed in dismantling the tissue stroma but in these studies they were avoided in order to prevent the possible proteolysis of components of interest. The use of primary cell cultures (as described in chapter 5) may in future work prove to be a more successful approach.

The isolation of membranes from primary tissue showed differences between the two assay methods, with 5'NT activity not corresponding to the qualitative or quantitative appearence of the membrane preparation by electron microscopy (figures 6.1 and 6.2.). This discrepancy may be due to, (i) the altered proportions of ER, Golgi, mitochondrial and plasma membrane in the preparations, not discernable by electron microscopy, or (ii) the inactivation of 5'NT activity. The inactivation of the enzyme may have resulted from a single step in the processing of the sample; the stainless steel probe of the rotary homogenizer may

have contributed to the loss in 5'NT activity by leaching metal ions and fragments into the tissue homogenate. Enzyme activity may also have been affected by trace metal elements in the sucrose used for the gradient separation, although this is less likely since a high grade sucrose was used ("Analar" grade, BDH, see appendix C).

The cell line membrane preparations as determined by electron microscopy alone, shows a wide variation in size of membrane fragments, which could be the result of many factors in the disruption technique, and the differential centrifugation protocol. The presence of a membrane stabilizer such as $ZnCl_2$ prior to homogenization has been used in previous studies (Perdue & Sneider, 1970; Brunette & Till, 1971; Gruber, *et al.*, 1984). The use of zinc as a stabilizer must be determined empirically for individual cell types, as some groups have found that excessive homogenization is required after pretreatment with zinc (Gruber, *et al.*, 1984). Excessive homogenization may result in the disruption of the nuclear envelope thereby contaminating the preparation with nucleic acids. Homogenization in the absence of $ZnCl_2$ is thought to yield a higher proportion of the plasma membrane as large fragments. Furthermore, the ommission of zinc from membrane preparations is thought to yield a higher contamination with ER membrane and mitochondria (Gruber, *et al.*, 1984). The considerable variation in size of membrane fragments from the cell line

preparations could also be due to the two-phase separation method, which is based on the chemical properties of the membranes rather than previous techniques which rely on differences in density and sedimentation. More uniformity in plasma membrane fragments could also be achieved by not pooling the P1, P2 and P3 fractions, but subjecting them seperately to the two-phase system.

The contamination of the T47D membrane preparation with mitochondria is either due to the absence of $ZnCl_2$ as a membrane stabilizer (as discussed above) or, the result of overloading the two-phase system. The latter is the more likely alternative as no such contamination was seen in the MCF-7 membrane preparation. The problems associated with overloading the two-phase system have also been identified in another study (Gruber, *et al.*, 1984) where they showed a decreased purity of the isolated plasma membrane. Overloading the system could give rise to the physical trapping of organelles, and other cell components, thereby preventing separation into the correct phase. The presence of large membrane sheets would be likely to contribute to this trapping problem.

The preliminary findings using the water solubility method of fractionation showed a number of cellular components separated and visualized using the silver stain reaction. The poor resolution and definition observed in the lower molecular weight regions in both water soluble and insoluble fractions

is due to overstaining with silver (necessary to visualize the components in higher molecular weight regions). The use of a gradient gel, in future studies, would enhance the definition/sharpness of the bands isolated, thereby reducing the possibility of silver grains coalescing between separated bands. The smearing observed in the water insoluble fraction could be due to a number of factors; (i) nucleic acids, or (ii) an insufficient SDS concentration in sample buffer resulting in inadequate solubilization of fractionated sample. Some studies have used nucleases to break down nucleic acids (Garrels, 1979), although this is only useful when detecting radiolabelled components, and may have further complications by the addition of contaminating proteases in the nuclease preparations. The presence of very high molecular weight components retarded by the stacking gel, particularly in the water soluble fraction may be glycolipids, high molecular weight mucins or aggregates not fully solubilized by SDS.

Although a large number of bands were separated, as determined by fluorography, there was considerable smearing and subsequent blackening of the X-ray film due to silver grain coalescence.

These approaches show a number of differences between the two fractions. Further studies using these methods, with the refinements discussed earlier, may prove to be successful in rapidly identifying the localization of the high molecular weight glycoproteins.

CHAPTER 7

CONCLUSIONS AND FUTURE STUDY.

There is now considerable evidence that malignant transformation is accompanied by many changes in the oligosaccharide structures of glycoproteins. The study has shown that there are significant differences in the nature of glycoproteins released from benign and malignant human breast tissue. This work on human primary tissue supports previous evidence, predominantly from animals and cell lines, with a number of glycoproteins being identified in tumour tissue. It was noted that the converse also occurred but to a much lesser extent.

It is not clear whether these glycoproteins, some of which appear to be tumour specific, are the result of *de novo* synthesis of protein/glycoprotein or abnormal glycosylation of an otherwise normal protein. The evidence from studies of MFGM, or polymorphic epithelial mucins (PEMs) as it is now referred to, using immunochemical and molecular biological approaches (Gendler, *et al.*, 1989), suggests that alterations of this nature are due to abnormal

glycosylation.

In the present study significant differences were identified in relation to the incorporation of [^{3}H]fucose, although it remains unclear whether this change is due to fucose *per se*. Increase in the size and branching of glycoproteins, a feature recognized with malignant transformation (Jarnefelt, *et al.*, 1978), could account for the changes observed by providing more sites for fucose binding.

The inhibition or alteration of the lysosomal enzyme alpha-L-fucosidase could result in the over-expression of fucose in complex glycoconjugates at the surface of tumour cells. A recent study assessing the characteristics of alpha-L-fucosidase in hepatocellular carcinoma showed a significant decrease of specific activity of the enzyme in tumour as compared to non-tumour tissue (Leray, et al., 1989).

It is more likely that alterations in specific transferase enzymes are responsible for the changes observed. Studies of chemically induced carcinogenesis in rat liver have shown a significant increase in activity of an alpha-fucosyl transferase (Holmes & Hakomori, 1983). Moreover altered Chinese hamster ovary cells have been shown to express a fucosyl transferase activity not detected in the parental CHO cells (Campbell & Stanley, 1983). If similar differences occur in human tumours a specific alteration of this type could result in the synthesis of fucose-glycoproteins specific to tumour cells. Evidence of altered levels of fucosyltransferase

activity between high and low metastatic rat cell lines has also been demonstrated (Chatterjee & Kim, 1978). This may explain the heterogeneity observed between tumours with regard the expression of the high molecular weight glycoproteins in the present study.

There is evidence from embryonic studies that there are changes in fucosylation of type 2 blood group chains at different stages of differentiation which depend on the relative activities of certain glycosyltransferases (Gooi, *et al.*, 1981). The changes occuring in malignancy may reflect these normal stages of differentiation during embryogenesis.

It has been postulated that a specific role exists for the fucosylated membrane glycoproteins in mammalian cells; the proximity of the fucose residue to the polypeptide core, may serve to bind proteins for membrane insertion or for transport through the membrane (Glick, 1978). Alterations in the incorporation of fucose could therefore have a significant effect on cell behaviour. However, the increase in fucose labelling observed in this study may be an effect and not a cause of aberant cell behaviour in malignancy. Unfortunately, the findings of this study do not clarify the situation, although future work on the cellular localization of the glycoproteins may "shed light" on a function indicating a probable cause or effect role.

The identification of the high molecular weight group of glycoproteins as membrane or secretory components is still not certain. However, the findings

do suggest that the glycoproteins are either secreted or membrane associated, and not integral membrane components since the labelling and turnover/secretion time is so rapid. Indeed, a number of earlier studies using animal cell lines have suggested that metastasizing tumours may shed glycocalyx into the serum (Kim, 1970; Kim, *et al.*, 1975; Chatterjee, *et al.*, 1976). Furthermore, the immunohistochemical findings of this thesis using the antiserum P5252, suggests that the glycoprotein of 230/250kD is localized predominantly at the luminal cell surface in structures showing such differentiation.

The multiplicity of conformations and energetically expensive biosynthesis of glycoproteins as a whole suggests that the glycoproteins have an as yet unidentified function rather than being mere elaborate cellular decoration.

The following conclusions have been made from the work described in the preceeding chapters concerning the analysis of glycoproteins released from the human breast:

(i). The fucosylated glycoprotein of 230kD is a novel tumour associated component, and not a differentiation antigen, released from malignant human breast tissue.

(ii). The high molecular weight glycoproteins of 230 and 250kD have the same or very similar protein core structure.

(iii). The high molecular weight glycoproteins of 230, 250 and 280kD are, or comprise a majority of N-linked glycan chains.

(iv). The polyclonal antiserum P5252 recognizes epitopes to the high molecular glycoproteins of 230 and 250kD.

(v). The expression of the high molecular weight group of glycoproteins in human tumour cell lines, in particular those lines derived from primary tumours and not metastases, parallels the findings in primary tissue.

The identification of an apparently novel group of glycoproteins, which are not detected by a number of established antisera and show an association with tumourigenicity, warrants further study to fully characterize their localization, structure and hence likely function. Future work would include the purification of the high molecular weight group of glycoproteins either by gel filtration or by affinity chromatography using the polyclonal antiserum P5252. Subsequent studies using the purified glycoproteins, would involve the deglycosylation, mapping and ultimate sequencing of the protein core structure to establish the identity or non-identity between the glycoproteins. Studies using antibodies against the deglycosylated glycoprotein could determine if the glycoproteins released from the carcinomas are formed *de novo* or whether they represent altered glycosylation of glycoproteins synthesized by normal

breast. Moreover, antibodies directed against the protein core, could be used to screen a cDNA library (derived from a human mammary carcinoma) in order to determine the similarity of the protein core sequence between the high molecular weight glycoproteins identified in the present study. This approach has been used in determining the nature of the high molecular weight mucin molecules of the HMFGM (Gendler, *et al.*, 1989).

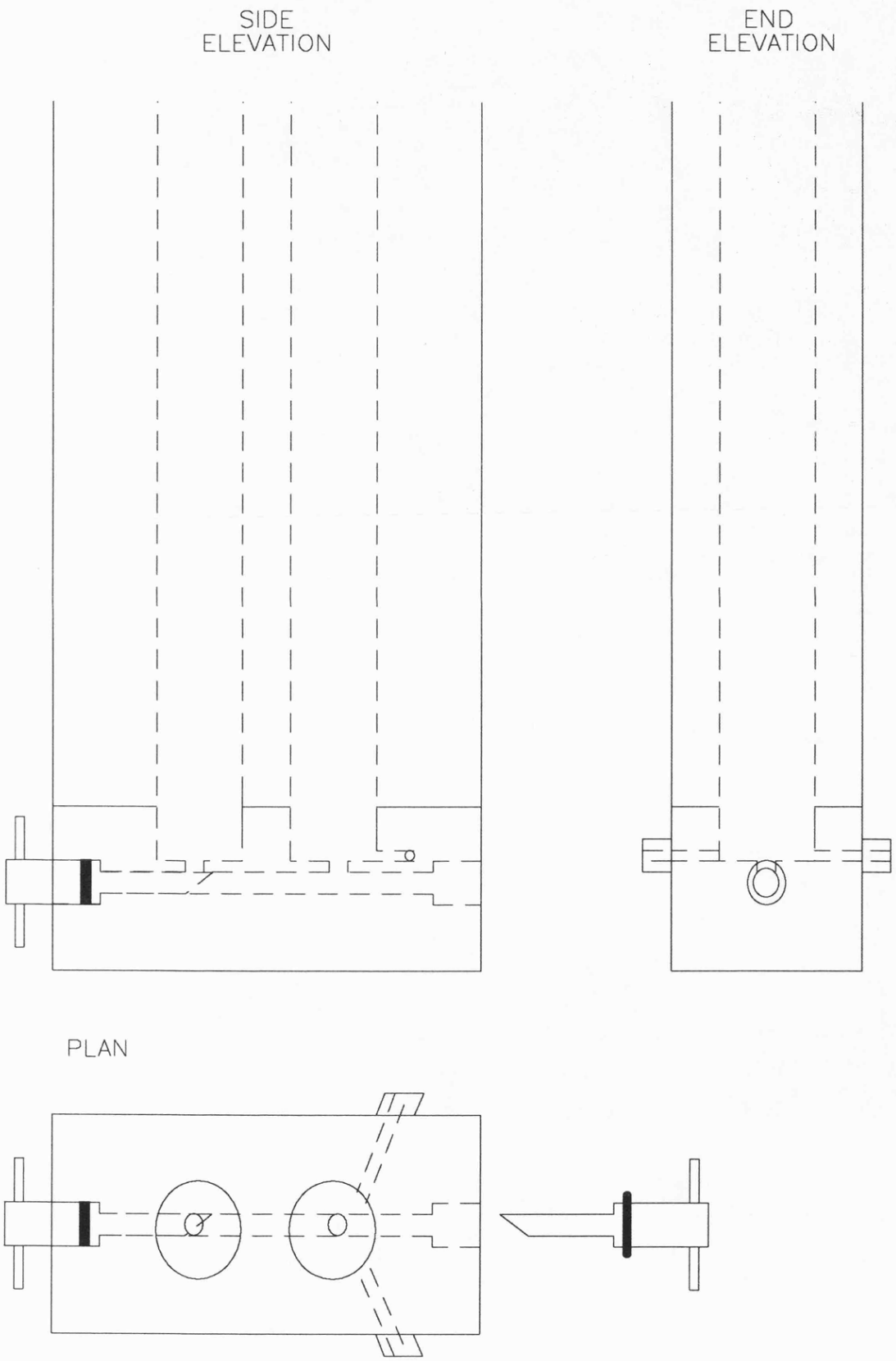
A detailed assessment of the carbohydrate composition and sequence could be done using commercially available exo- and endo-glycosidases with the techniques of both Western blottting and HPLC. The effects of glycosylation inhibitors such as castanospermine and swainsonine could also add to the information about these glycoproteins enabling more accurate speculation as to their role in the development of breast cancer. A continuation of the studies outlined in chapter 6 concerning the localization of the glycoproteins of interest, may also help in the identification of their role in this disease.

Work, by the author, is currently in progress to raise both polyclonal and monoclonal antibodies to epitopes on these high molecular weight glycoproteins in order to realise the potential prognostic value of these glycoproteins in determining the progression of this disease. Further study of the effects of modulating or differentiating agents on these glycoproteins could clarify the relationship with

tumour progression and may even modify tumour cell behaviour. Hence, this interesting biochemical finding may prove to be of relevance in the understanding and management of what is a common female cancer.

APPENDICES

Figure A.1. Linear gradient gel pouring apparatus.



APPENDIX A: GEL APPARATUS

Linear gradient gel pouring apparatus.

This apparatus was designed by the author to assist in the accurate and reproducible pouring of linear gradient gels. The bulk of the apparatus was made of perspex and the taps of stainless steel. The design is shown in figure A.1.

APPENDIX B: BUFFERS

Phosphate buffered saline, (PBS).

This was a standard buffer solution used in a number of protocols. Concentrated buffer solution (500ml aliquots) comprising 0.58M sodium chloride, 0.03M disodium hydrogen orthophosphate and 9.9mM sodium dihydrogen orthophosphate were prepared. Each 500ml aliquot of this concentrate was diluted to 8l using reverse osmosis treated water to provide a working buffer solution of pH 7.2.

Acetate buffer (section 2.4.3).

Stock solutions of 0.2M acetic acid (solution A) and 0.2M sodium acetate (solution B) were prepared seperately. A buffer of pH 5.4 was made by adding 8.8ml of A to 41.2ml of B and made up to 100ml with ultra pure water after checking the pH.

Barbitone buffer (section 4.2.4).

Barbitone buffer comprising 0.058M sodium barbitone (5'5 diethylbarbituric acid sodium salt) and 0.024M barbitone (5'5 diethylbarbituric acid) was prepared and adjusted to pH 8.2. A preservative, merthiolate (0.15g), was added and the volume made up to 1l. This buffer was used in the preparation of the 2% barbitone agar solution described in section 4.2.4. The addition of a preservative was necessary as the barbitone agar could not be autoclaved.

"Karnovsky" fixative (section 6.1.3).

This solution, used for the fixation of material prior to processing for electron microscopy, contains freshly prepared paraformaldehyde and gluteraldehyde. A 4% solution of paraformaldehyde was made by dissolving 20g of the dry powder in 250ml ultra pure water and heating to 90-95°C. A few drops of 1N NaOH was added slowly with stirring in order to clear the solution. After cooling, 100ml of 25% gluteraldehyde was added and the mixture made up to 500ml with 0.1M PBS (pH 7.4-7.6). The final solution was then adjusted to pH 7.2.

Cacodylate buffer (section 6.1.3).

This buffer was prepared by adding 2.7ml 0.2M HCl to 50ml 0.2M sodium cacodylate and making up to 200ml with ultra pure water. This solution was used as a general wash solution in the processing of material for electron microscopy.

APPENDIX C: REAGENT AND EQUIPMENT SOURCE LIST

Chemical Reagents.

All chemicals unless otherwise stated below, were purchased from BDH Chemicals Ltd., Poole, UK. Electran grade chemicals were used in the preparation of reagents for electrophoresis. Where appropriate, "analar" grade chemicals were used.

Name: SDS-6H

Composition: Carbonic anhydrase, 5 mg/vial
Ovalbumin, 25 mg/vial
Bovine albumin, 25 mg/vial
Phosphorylase B, 0.5 mg/vial
B-Galactosidase, 0.5 mg/vial
Myosin, 0.5 mg/vial

Supplier: Sigma Chemical Company Ltd.
Fancy Road
Poole
Dorset
BH17 7NH
U.K.

Enzymes.

Name: 5'-Ribonucleotide
phosphohydrolase,

(E.C. No.3.1.3.5)

Source: *Crotalus atrox* Venom

Conc.: 538 Units/mg protein,
70 Units/mg solid

Unit Def.: One unit will hydrolyze 1.0 uMole of Adenosine 5'-monophosphate per minute at pH 9.0 at 37°C.

Supplier: Sigma Chemical Company Ltd.
Fancy Road
Poole
Dorset
BH17 7NH
U.K.

Radiochemicals.

All radiolabelled chemicals were obtained from Amersham International Plc., White Lion Road, Amersham, Buckinghamshire, HP7 9LL, U.K.

Name: [2-^{3}H] Adenosine 5'-monophosphate, ammonium salt.

Specific activity: 20 Curies/mmol,57.5mCi/mg

Radioactive conc.: 1 mCi/ml

Name: L-[6-^{3}H] Fucose

Specific activity: 70 Curies/mmol,414 mCi/mg

Radioactive conc.: 1.0 mCi/ml

Name: D-[6-^{3}H] Galactose

Specific activity: 31.5 Curies/mmol,173mCi/mg

Radioactive conc.: 1.0 mCi/ml

Name: D-[6-^{3}H] Glucosamine hydrochloride

Specific activity: 27 Curies/mmol,124 mCi/mg

Radioactive conc.: 1.0 mCi/ml

Name: L-[4,5-^{3}H] Leucine

Specific activity: 130 Curies/mmol,928 mCi/mg

Radioactive conc.: 1.0 mCi/ml

Name: ^{35}SLR, general purpose
^{35}S-labelling reagent

Specific activity: 1240 Curies/mmol

Radioactive conc.: 1.0 mCi/ml

Immunological Reagents.

Antibodies were obtained from a number of sources;

Primary Antibodies;

Name: BRST-1 (or CU-18)

Source: Mouse ascites fluid

Subclass: IgG_1

Antibody concn.: 0.2mg/ml

Specificity: BRST-1 detects BCA-225, a glycoprotein secreted by the T47D brest carcinoma cell line. Strong intracytoplasmic staining is seen in primary and metastatic breast carcinoma tissue, as well as in cervical carcinomas. Apical/glycocalyx staining is seen in normal kidney, lung, fallopian tube, liver, skin (eccrine sweat glands) and uterus. Similar staining patterns are observed in lung, ovarian and endometrial cancers. Carcinomas of the colon, stomach, prostate, bladder, liver, pancreas, thyroid and parotid are negative, as are sarcomas and lymphoid cancers.

Supplier: Cambridge Research Laboratory
Research Products Group
195 Albany Street
Cambridge
Massachusetts 02139
USA.

Name: HMFG1

Source: Clone 1.10.F3

Subclass: IgG_1

Specificity: Reacts with breast epithelium in paraffin and frozen sections. Binds strongly to components of HMFG.

Name: HMFG2

Source: Clone 3.14.A3

Subclass: IgG_1

Specificity: As above.

Supplier: Oxoid Ltd
Wade Road
Basingstoke
Hampshire
RG24 0PW
U.K.

Name: Anti-bromodeoxyuridine

Source: clone Bu20a

Subclass: IgG_1, kappa

Antibody concn.: 171ug/ml

Specificity: Identifies bromodeoxyuridine (BrdU) in single stranded DNA, free BrdU, or BrdU coupled to a protein carrier. There is minimal cross-reactivity with other nucleosides, e.g. thymidine.

Supplier: Dakopatts Ltd.
22 The Arcade
The Octagon
High Wycombe
Buckinghamshire
HP11 2HT
U.K.

Secondary Antibodies;

Rabbit anti-mouse immunoglobulin antisera, peroxidase labelled.
Swine anti-rabbit immunoglobulin antisera, peroxidase labelled.
Swine anti-rabbit immunoglobulin antisera, unlabelled.

Supplier: Dakopatts Ltd.
22 The Arcade
The Octagon
High Wycombe
Buckinghamshire
HP11 2HT
U.K.

Lectins;

Name: Wheat Germ Agglutinin, peroxidase VI labelled

Source: *Triticum vulgaris*

Specificity: B-D-GlcNAc(1-4)-B-D-$GlcNAc_3$, (1-4)-B-$DGlcNAc_2$, sialic acid

Name: UEA-I, peroxidase VI labelled

Source: *Ulex europaeus*

Specificity: a-L-Fucose $(D\text{-}GlcNAc)_2$

Name: Peanut agglutinin, peroxidase VI labelled

Source: *Arachis hypogaea*

Specificity: D-Gal-B-(1-3)-GalNAc

Supplier: Sigma Chemical Company Ltd.
Fancy Road
Poole
Dorset
BH17 7NH
U.K.

Major Equipment suppliers.

Beckton Dickinson UK Ltd., Falcon organ culture dishes
Between Towns Road,
Cowley,
Oxford,
OX4 3LY
U.K.

Biorad Laboratories Ltd., Caxton Way, Watford, Hertfordshire, U.K.	Gel drier and blotting cassette
BOC Ltd., Deer Park Road, London, SW19 3UF U.K.	Special Gases
Corning Ltd., Stone, Staffordshire, ST15 0BG U.K.	pH meter
Elga Ltd., Lane End. High Wycombe, Buckinghamshire, HP14 3JH U.K.	Water purification system Cartridges; SC31, SC6, SC1.
Envair (UK) Ltd., York Avenue, Broadway Industrial Estate, Haslingdon, Rossendale	Class II microbiological safety cabinet

Lancashire,
BB4 4HX
U.K.

Fisons Scientific Apparatus, -70.C freezer
Bishop Meadow Road,
Loughborough,
Leicestershire,
LE11 0RG
U.K.

Jencons Scientific Ltd., Cryogenics
Cherrycourt Way Ind. Est.
Leighton Buzzard,
Bedfordshire,
U.K.

Kodak Ltd. X-ray film processor
PO Box 66,
Kodak House,
Station Road,
Hemel Hempstead,
Hertfordshire,
U.K.

Kontron Instruments Ultra centrifuge
Bernstrasse Sud 169, Centrikon T-2070
CH-8010 Zurich,
Switzerland.

LEEC Ltd., CO_2 incubator
Private Road No.7,
Colwick,
Nottingham,
NG4 2AJ
U.K.

Leitz Instruments Ltd., Microscope
Luton,
Bedfordshire,
U.K.

MSE Scientific Instruments, Centrifuge
Sussex Manor Park, Europa 24M
Gatwick Road,
Crawley,
West Sussex,
RH10 2QQ
U.K.

Pharmacia LKB Ltd., Chromatography accessories
Pharmacia House,
Midsummer Boulevard,
Milton Keynes,
Buckinghamshire,
MK9 3HP
U.K.

Laboratory Thermal Equip., Autoclave 225E
Greenfield,

Oldham,
Lancashire
U.K.

Reichert-Jung UK Ltd., 820 Yeovil Road, Slough, Berkshire, U.K.	Microtome
Shandon Southern Ltd., Frimley Road, Camberley, Surrey, U.K.	Cytospin
Sterilin Ltd., 43-45 Broad Street, Teddington, Middlesex, TW11 8QZ U.K.	Tissue culture plastics

APPENDIX D: PHOTOGRAPHIC TECHNIQUES

Development of fluorographs.

After exposure of the X-ray film at -70°C the film was allowed to regain room temperature before developing in a Kodak X-omat automatic developer (by kind permission of Department of Medical Physics, Leicester Royal Infirmary).

Black and white photography.

All the black and white photographs in this thesis were taken developed and printed by the author. Film type and development was as follows:

35mm film; Kodak Panatomic-X, 32 ASA, developed for 6 minutes at 20.C using D-76 (undiluted), washed for 1 minute, fixed for 10 minutes in rapid fixer (Kodak) washed for 20 minutes in running tap water at 20.C, before finally rinsing in Photo-Flo (Kodak) and drying.

Sheet film; Kodak T-Max, panchromatic sheet film (8"x10"). This film was used to obtain full size reproductions of polyacrylamide gels stained by the silver staining technique. Film exposure was determined empirically each time but varied between 2-3 seconds. Tray development using D-76 (Kodak) was for 7 minutes at 20°C, fixed for 10 minutes in rapid fixer and finally washed for 20 minutes in running tap water at 20°C.

Colour photography

Colour film for photomicrographs was taken using Ektachrome colour reversal film, 50 ASA. Development of the films was by a local commercial firm.

APPENDIX E: PURIFICATION OF ENDO F

Due to the high cost of commercially available endoglycosidases an attempt was made to purify sufficient quantities for the further analysis of the high molecular weight glycoproteins identified. The experimental procedure followed exactly the method outlined by Elder & Alexander (1982). Unfortunately separation was unsatisfactory as determined by SDS-PAGE.

Bacterial culture: *Flavobacterium meningoscepticum* was used as a source for the endoglycosidase F. This aerobic, non-motile, gram-negative rod (Bergey) is known to secrete the enzyme endo-B-N-acetylglucosaminidase F when grown in culture. Although it is well recognized that infection with *F. menigoscepticum* causes neonatal meningitis (King, 1959), with a 55% mortality rate, it is rarely a causative agent in adults. Nevertheless one of the least virulent serotypes was used. The organism was obtained from Professor Sneath of the Department of Microbiology at the University of Leicester.

Genus;	*Flavobacterium*
Species;	*menigoscepticum*
ATCC no.	13253
Strain;	14
Type;	A
NCTC no.;	10016

Univ. no.; D1979

(ATCC-American Type Culture Collection, Rockville, USA).
(NCTC-National Collection of Type Cultures, London, UK).

The type and strain were confirmed by two API tests carried out by Mr. P. Clayton of the PHLS laboratories at Leicester Royal Infirmary.

The organism was supplied and maintained as a glass bead culture stored at -70°C. These cultures were prepared by resuspending pure colonies in 1ml nutrient broth containing 15% glycerol and 20-30 sterile 2mm glass beads (Feltham *etal.*, 1978). This bead culture was then mixed and frozen at -70°C. When fresh pure cultures were required a glass bead was chipped off and used to inoculate a nutrient agar plate or broth.

Four 2l flasks each containing 1l of M9 minimal salts medium were inoculated with overnight cultures of *F. meningoscepticum* in 10ml nutrient broth. Cells were grown in a 37°C shaking incubator at 300 revs./minute for 10 hours to reach stationary phase, as determined by plotting a calibration curve of absorbance (600nm) verses time for a previous "dry" run (figure A.2).

Purification: Cells were separated and discarded from centrifugation at 5000xg for 10 minutes. The

Figure A.2. Bacterial growth as a function of optical density versus time.

Time (hrs)	Mean O.D. 600nm
2	0.005
4	0.086
5	0.158
6	0.266
8.25	0.525
9.75	0.532
11.25	0.533

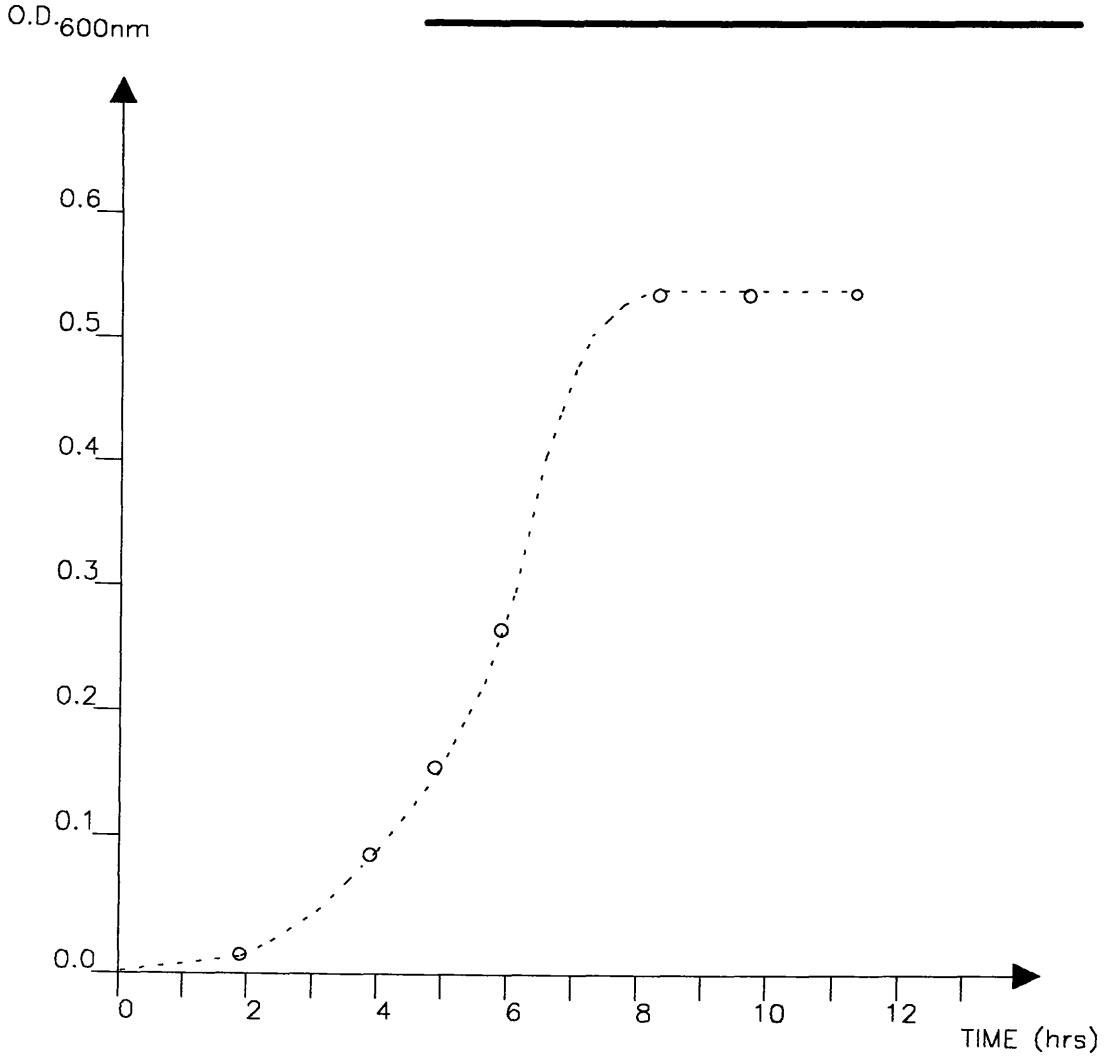


Figure A.3. Purification of an endoglycosidase from *Flavobacterium menigoscepticum*.

Chart speed 0.5mm/min
Fraction size 2.5ml
Void volume V_0 7.5ml
Column flow rate 7.2ml/hr
Column size 1.5x50cm
Detection wavelength 254nm

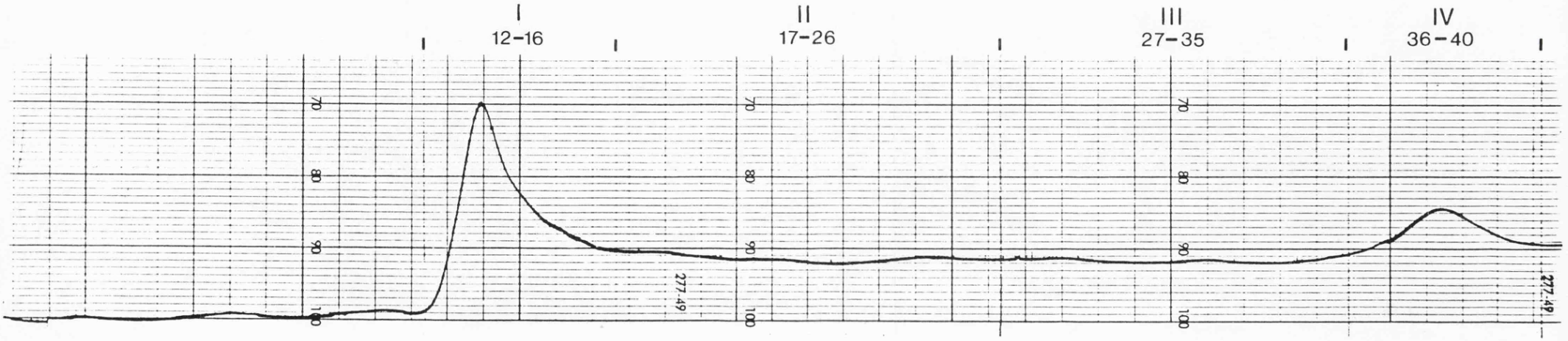
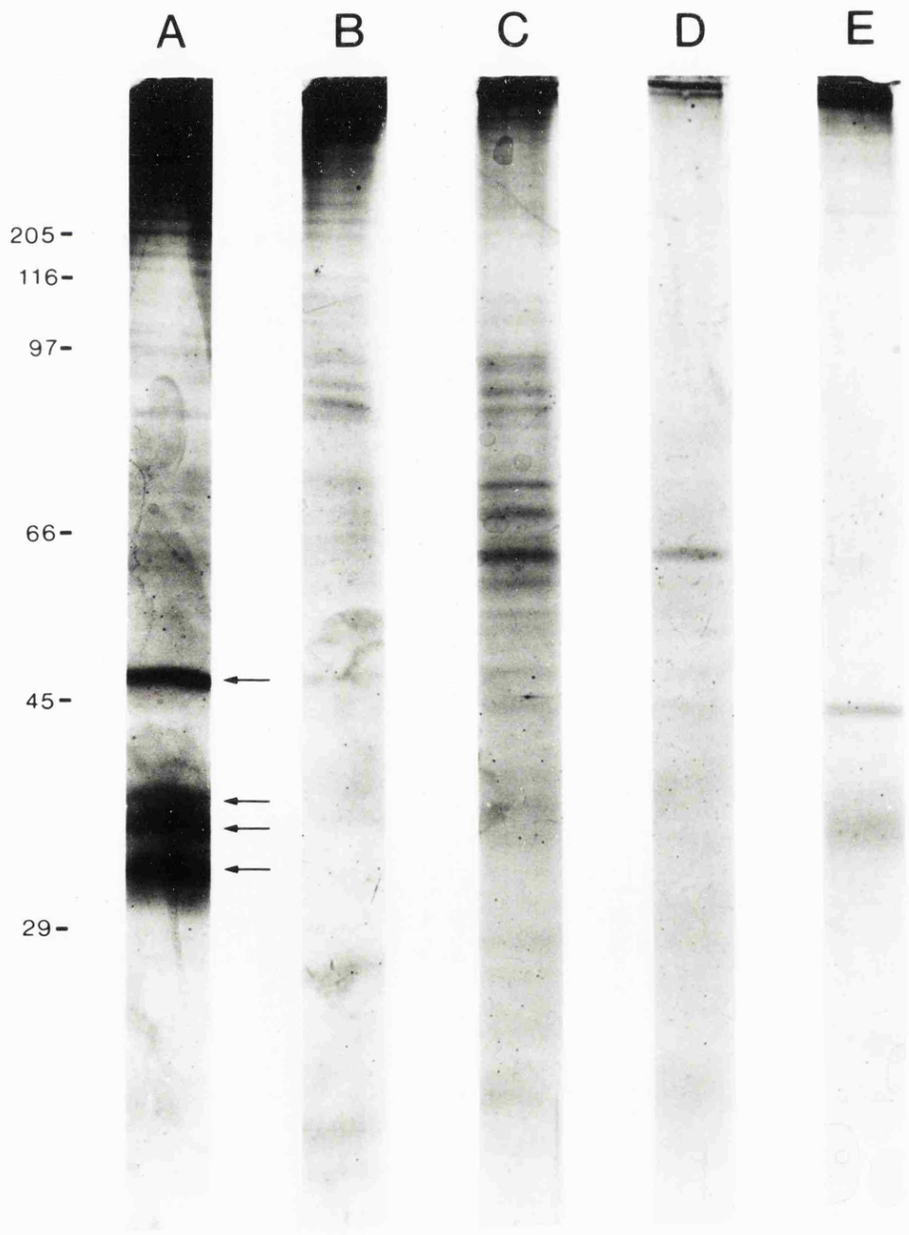


Figure A 4. SDS-PAGE of crude enzyme preparation (A), and pooled column fractions I-IV (B-E). Arrows highlight the four bands referred to by Elder and Alexander (1982).



supernatant was concentrated by addition of 2.244kg of ammonium sulphate giving 80% saturation. After stirring for 1 hour at 4°C the solution was centrifuged at 10000xg for 20 minutes. The pellet was resuspended in 50% ammonium sulphate, recentrifuged and the final pellet dissolved in 4ml of sodium phosphate buffer (pH 7.2) containing 0.01M sodium phosphate, 0.15M sodium chloride and 50mM EDTA. The sample was finally run on a 1.5x50cm ACA54 column at 4°C in the sodium phosphate buffer containing 5mM EDTA. The column flow rate was set at 7.2ml/hr and 2.5ml fractions were collected.

Detection of peaks by UV (254nm) revealed only two peaks, one at fraction no.s 12-16, the other at fractions 36-40,(figure A.3). As a result it was decided to pool fractions from the column run as follows;

fraction no.	pooled fraction no.
12-16	I
17-26	II
27-35	III
36-40	IV

These pooled fractions were desalted and concentrated using Amicon centrifugal concentrators (10000 MW cut off), to a final volume of about 1ml and 1mM (final concentration) PMSF was added. These fractions were separated on SDS-PAG and silver stained (figure A.4).

Results: The pre-column extract (lane A) shows a

number of bands in particular a group of four strongly staining bands between 30-50kD. However these bands are virtually absent from the fractions I-IV (lanes B-E).

Discussion: The 3-4 stongly staining bands present almost exclusively in the pre-column extract, could be the four Coomassie Blue staining bands briefly refered to in Elder & Alexander's paper. They suggested that the endoglycosidase activity was present in one or all of these bands. The absence of these major bands in the separated fractions I-IV indicate that they are either bound to the column or that they are retarded beyond the time limits set for the separation. It was therefore decided to repeat this experiment, at a later date, using the technique of size exclusion HPLC.

Minimal salts medium M9.

The following components; 40mM di-sodium hydrogen orthophosphate, 22mM potassium di-hydrogen orthophosphate, 9mM sodium chloride, and 19mM ammonium chloride, were made up in 4l ultra pure water, adjusted to pH 7.4 and autoclaved. Separate aliquots of 1M magnesium sulphate (8ml) and 1M calcium chloride (0.4ml) were also autoclaved and added to the cooled medium with 40ml 20% filter sterilized glucose. The medium was further supplemented with meat peptone no.70 (2.5g/l).

Nutrient agar/broth.

To 900ml of ultra pure water was added 3g beef extract, 5g peptone no.70, and 15g agar (omit for broth). The solution was adjusted to pH 6.8-7.0, heated in a waterbath to dissolve the constituents and finally made up to 1l with ultra pure water. For preparation of broth the mixture was decanted in 15ml aliquots prior to autoclaving. For nutrient agar the mixture was autoclaved and allowed to cool to 50°C before pouring plates. Poured plates were kept at 4°C and dried breifly at 37°C immediately before use.

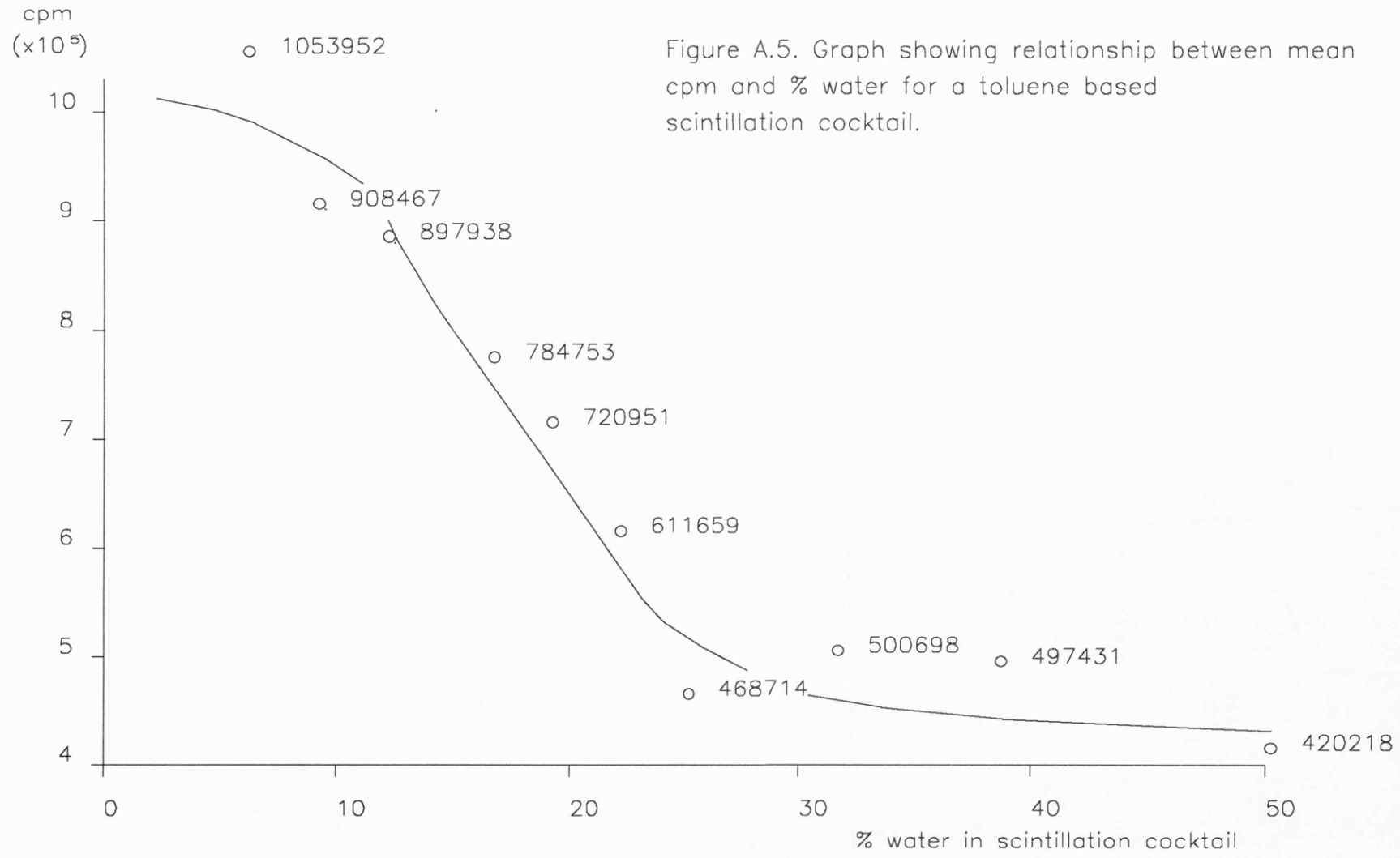


Figure A.5. Graph showing relationship between mean cpm and % water for a toluene based scintillation cocktail.

APPENDIX F: SCINTILLATION FLUID

Interfacing experiment, (section 6.1.4).

This assay was done to assess any interfacing effects in the scintillation fluid used. To each of 12 vials 1uCi [^{3}H] AMP was added and allowed to dry. Scintillation fluid containing from 0-5% water was added to each vial, mixed for 15 minutes on an orbital shaker and left to stand at room temperature for 24 hours before counting. The relationship between percentage of water present and cpm values are shown in figure A.5.

From these findings the volume of sample, from the 5'NT assay, to be added to the scintillation cocktail was determined.

APPENDIX G: PUBLISHED AND PRESENTED MATERIAL

Abstracts and poster presentations.

1. Walker R.A. & Rye P.D. Analysis of glycoproteins of non-malignant and malignant human breast. (J. Pathol. 1987, 151, 40a).
Poster at; Pathological Society, London, January 1987.

2. Rye P.D. & Walker R.A. Altered expression in vitro of human breast tumour associated glycoproteins by hormone additives.
Poster at; Biochemical Society, Nottingham, July 1988.

3. Rye P.D. & Walker R.A. Expression and modulation of breast tumour associated glycoproteins. (J. Pathol. 1989, 157, 161A).
Poster at; Pathological Society, London, January 1989.

4. Corcoran D., Rye P.D. & Walker R.A. Isolation and culture of cells from human primary breast carcinomas. (J. Pathol. 1989, 157, 163A).
Poster at; Pathological Society, London, January 1989.

5. Rye P.D. & Walker R.A. Heterogeneity of fucosylation pattern in high molecular weight glycoproteins from six human breast cancer cell lines.
Poster at; Xth International symposium on glycoconjugates, Jerusalem, September 1989.

Oral presentations.

1. Rye P.D. & Walker R.A. Altered expression in vitro of human breast tumour associated glycoproteins by hormone addition. Biochemical Society, Nottingham, July 1988.

2. Rye P.D. & Walker R.A. Expression and modulation of breast tumour associated glycoproteins. Pathological Society, London, January 1989.

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1. Rye P.D. & Walker R.A. Altered expression in vitro of human breast tumour associated glycoproteins by hormone additives. Biochem. Trans. 1988, 16, 1026-1027.

2. Rye P.D. & Walker R.A. Analysis of glycoproteins released from benign and malignant human breast: changes in size and fucosylation with malignancy. Eur. J. Cancer Clin. Oncol. 1989, 25, 65-72.

3. Walker R.A. & Rye P.D. Fucosylation pattern of high molecular weight glycoproteins in six human breast cancer cell lines. (manuscript submitted).

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Analysis of Glycoproteins Released from Benign and Malignant Human Breast: Changes in Size and Fucosylation with Malignancy

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Abstract—*Radiolabelled glycoproteins released into media from benign and malignant human breast tissue after 48 h organ culture have been analysed using SDS polyacrylamide gel electrophoresis and fluorography.*

Major differences were identified between benign and malignant tissues: (i) a considerably higher incidence of glycoproteins in the molecular weight range 210–280 kDa detected from carcinomas than benign samples, with incidence being greater in poorly differentiated tumours; (ii) fucosylation of these glycoproteins released from carcinomas but not benign breast; (iii) heterogeneity, particularly of fucosylation, between the carcinomas but consistency amongst benign breast.

A glycoprotein of MW 230 kDa was of particular interest since it was not detected from any benign samples but was present in 11 of 17 carcinomas, when it was almost always fucosylated. This could prove to be a useful tumour marker.

INTRODUCTION

THERE IS ample evidence that modifications in the glycosylation of cell surface glycoproteins and glycolipids are associated with malignant transformation of cells [1–3]. Such alterations could be of significance in determining tumour cell behaviour since cell surface glycoconjugates have been implicated in cell–cell and cell–substrate interactions [4]. Many of the studies which have examined glycoprotein structure and synthesis of human tumours have been confined to malignant cell lines [5–7], rather than primary neoplasms, and comparisons with normal cells has been limited.

The identification of glycosylation changes in human breast carcinomas could be of importance with regard to the recognition of tumour associated markers and in the prediction of tumour behaviour. Previous histochemical studies using lectins confirmed that there are differences in specific sugar groups between the glycoproteins of benign and malignant human breast and that some alterations are related to tumour differentiation or to some extent metastatic potential [8–11]. Heterogeneity in the *in vitro* utilization of radioactive labelled sugars by malignant human breast in comparison to normal has also been found by using tissue autoradiography [12]. However, these histological approaches cannot give information about differences in glycoprotein structure and size occurring with malignancy. In the present study we have analysed the nature of glycoproteins released by a range of benign and malignant breast lesions during *in vitro* labelling with radioactive labelled sugars, using SDS polyacrylamide gel electrophoresis and fluorography. The results have been related to tumour characteristics such as differentiation and metastasis.

MATERIALS AND METHODS

Tissues

All breast tissues were obtained immediately after surgery. Approximately 1 g from each specimen was placed in 10 ml Eagles Minimal Essential Medium containing 200 U/ml penicillin and 200 μg/ml streptomycin and kept at 4°C until prepared for culture. Adjacent slices were fixed in 4% formaldehyde in saline and processed to paraffin wax. A total of six samples of benign proliferative disease, four fibroadenomas, 18 carcinomas and lymph node metastases for two of the carcinomas have been studied.

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Reagents

Dulbecco's Modified Eagles medium with L-glutamine (DMEM), Eagles Minimal Essential medium (MEM) and penicillin streptomycin were from Gibco; organ culture dishes were from Falcon.

D-[6-^{3}H]Galactose (specific activity 31 Ci/mmol) and L-[6-^{3}H]fucose (specific activity 70 Ci/mmol) were obtained from Amersham International plc.

Electran grade acrylamide, *N,N'*-methylene-bis-acrylamide, sodium dodecyl sulphate, ammonium persulphate and *N,N,N',N'*-tetra-methylethylene-diamine (TEMED) were from BDH Chemicals Ltd. Molecular weight markers for SDS-PAGE (MWSDS-6H), riboflavin, phenylmethylsulphonyl fluoride (PMSF) and Coomassie Brilliant Blue R-250 (CBR-250) were obtained from Sigma Ltd.

Organ culture

Tissues were diced from 1 mm cubes using opposing sterile skin graft blades with dental wax as a cutting surface. The raft culture method [13] was employed, with tissues placed on defatted lens tissue supported by stainless steel grids at the gas/liquid interface of organ culture dishes. Tissues totalling 100–130 mg wet wt were used per dish with 1.5 ml of medium, all tests being performed in duplicate. DMEM with L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin was used throughout with no other additives. After a 2 h pre-incubation the medium was changed, and 20 μCi/ml of [^{3}H]-fucose or galactose added. For three samples of benign proliferative breast, two fibroadenomas and six carcinomas, incubations were in an atmosphere of 95% air/5% CO_2 at 37°C for 18 and 48 h. The remaining samples were incubated for 48 h only. The medium was collected and the protease inhibitor PMSF was added to each sample to a final concentration of 1 mmol/l prior to storage at −20°C until required. The cultured tissue was fixed and processed for assessment of viability.

Electrophoresis

Medium from each incubation was dialysed and concentrated. Total protein levels were assayed using the method of Bradford [14]. Samples were denatured for 3 min at 100°C in the presence of 5% SDS and 2% mercaptoethanol. For each specimen samples containing 100 μg total protein were applied to 10–20% linear gradient polyacrylamide slab gels and run using the discontinuous buffer system of Laemmli [15]. Comparisons of individual sugar incorporation by benign and malignant cultures were performed on the same gel, using both 7.5% and 10% polyacrylamide gels. Electrophoretic runs were calibrated with a high molecular weight standard mixture containing myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). The gels were stained with CBR-250 and destained in a solution containing methanol/acetic acid/water (5:5:2 v/v). Calibration curves were plotted for each gel and the molecular weights of sample glycoproteins estimated by their relative mobilities.

Fluorography

After staining the gels were rinsed for 2 min in ultra pure water to remove excess acetic acid which would otherwise precipitate the fluor. They were then soaked in 300 ml of 1 M sodium salicylate for 30 min [16], immediately dried down and exposed to X-ray film (Fuji RX) for 14 and 21 days at −70°C. The resultant fluorograms were developed and scanned using a laser densitometer (LKB Ltd.).

Histology

Haematoxylin and eosin stained sections of the benign samples were assessed for the degree of proliferative change and of the carcinomas for type, using WHO criteria, and grade applying the Bloom and Richardson criteria with modification [17].

RESULTS

Comparisons of the incorporation of [^{3}H]sugars were made between (i) all benign specimens, (ii) carcinomas, (iii) benign and malignant samples and (iv) within individual cases. All comparisons were undertaken on samples analysed under the same percentage gel and electrophoresis conditions.

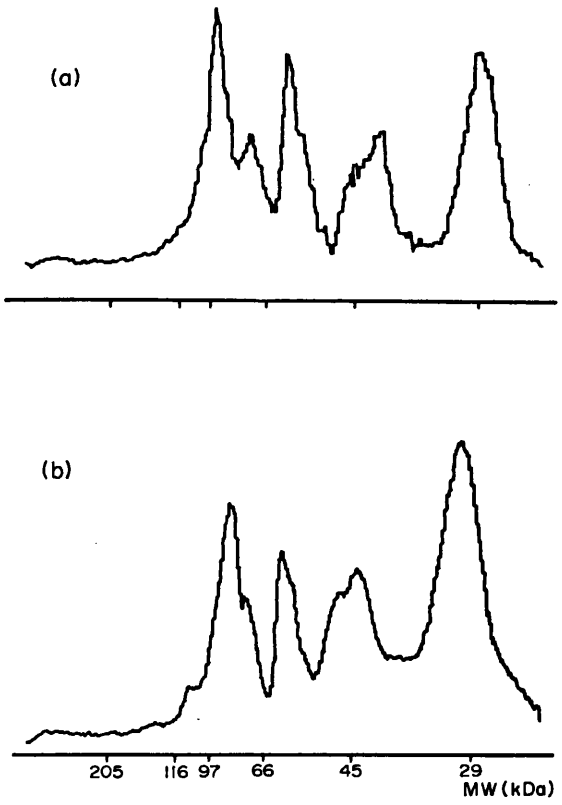
Comparison between 18 h *and* 48 h *cultures*

There was a similar distribution of glycoproteins identified from the 18 h and 48 h cultures for each of the samples tested (Fig. 1). The differences and similarities between the benign and malignant cases (see below) were evident after 18 h incubation.

There was no significant differences in the viability of tissues incubated for the two time periods, and the viability of all tissues incubated for 48 h was good, as assessed by morphology.

Comparison between benign tissues and carcinomas

Major differences were identified between benign and malignant specimens in glycoproteins (gp) of molecular weight greater than 200 kDa and in the incorporation of [^{3}H]fucose. Four gps in the MW range 210–280 kDa were identified in different combinations from all carcinomas whereas media from only four of the 10 benign samples contained gps within this range (Tables 1 and 2). One fibroadenoma released a glycoprotein of MW 210 kDa which showed weak incorporation of [^{3}H]-fucose and galactose (Fig. 2) but there was no evidence of [^{3}H]fucose incorporation into the higher molecular weight gps released from other benign tissues (Figs. 3 and 4). In comparison, there was a



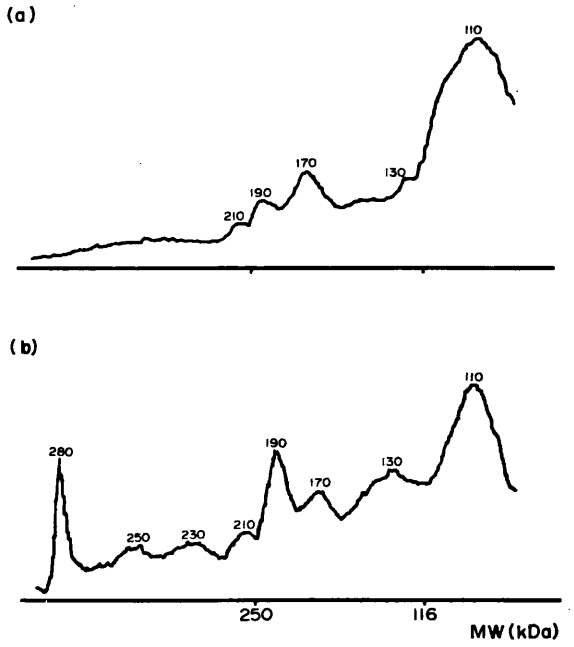
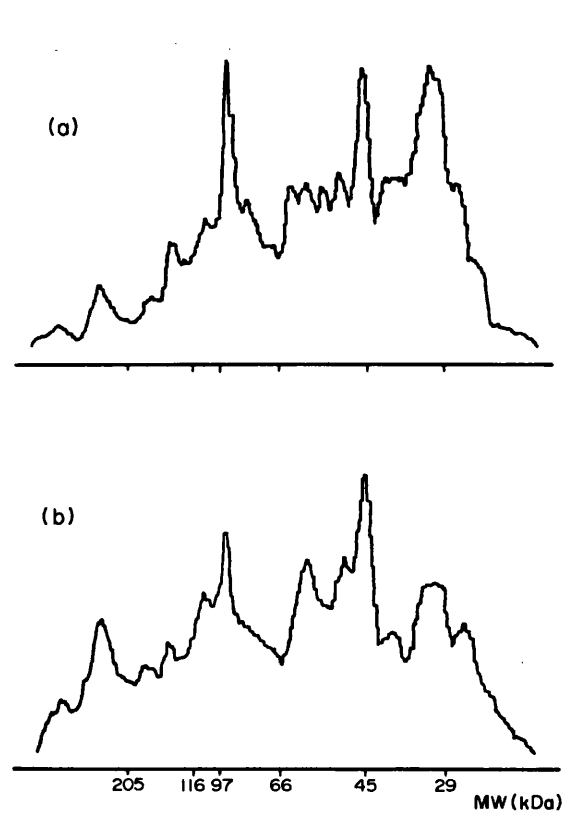


Fig. 2. Densitometric profiles of fluorograms of [^{3}H]*fucose labelled glycoproteins greater than* 100 kDa. 10% *SDS-PAGE. (a) Fibroadenoma (case* 3). *(b) Poorly differentiated carcinoma (case* 4).



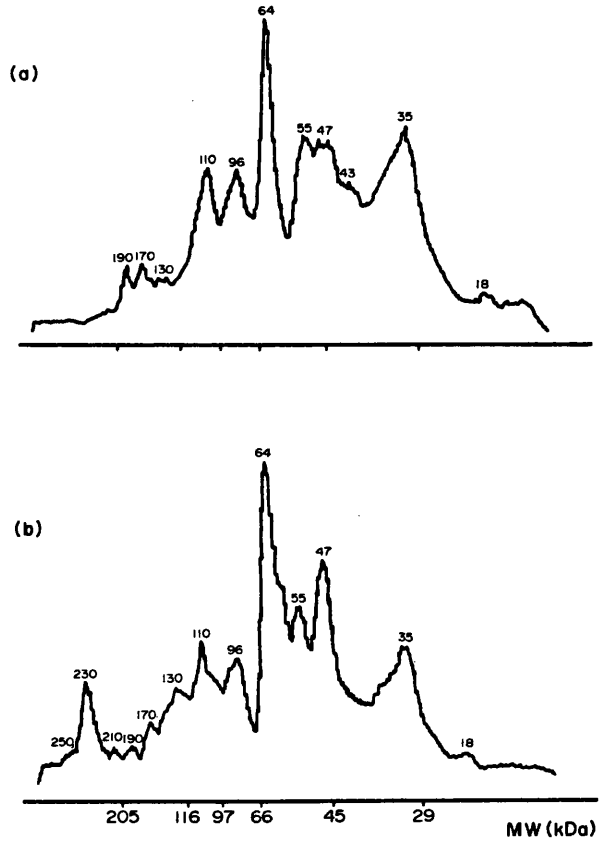


Fig. 1. A. Densitometric profiles of fluorograms of [^{3}H]*fucose labelled glycoproteins released from benign hyperplastic breast.* 10–20% *SDS-PAGE. (a)* 18 h *culture; (b)* 48 h *culture. B. Densitometric profiles of fluorograms of* [^{3}H]*fucose labelled glycoproteins released from a carcinoma.* 10–20% *SDS-PAGE. (a)* 48 h *culture; (b)* 18 h *culture.*

Fig. 3. Densitometric profiles of fluorograms of [^{3}H]*fucose labelled glycoproteins.* 10% *SDS-PAGE. (a) Benign hyperplastic breast (case* 9). *(b) Poorly differentiated carcinoma (case* 10).

Table 1. *The detection of glycoproteins* 210 kDa, 230 kDa, 250 kDa *and* 280 kDa *in media from cultured benign breast tissue*

Case No.	Histological features	Glycoprotein 210	230	250	280
1	Fibroadenoma	–	–	–	–
2	Fibroadenoma	–	–	–	–
3	Fibroadenoma	±FG	–	–	–
4	Fibroadenoma	–	–	–	–
5	Relatively normal	–	–	–	–
6	Focal mild hyperplasia	–	–	–	–
7	Diffuse mild hyperplasia	–	–	–	–
8	Diffuse mild hyperplasia	–	–	+G	+G
9	Focal moderate hyperplasia	+G	–	–	–
10	Diffuse moderate hyperplasia	+G	–	–	+G

– = absent; + = present; ± = weakly present; G = galactose; F = fucose.

Table 2. *The detection of glycoproteins* 210 kDa, 230 kDa, 250 kDa *and* 280 kDa *in media from cultured breast carcinomas*

Case No.	Type	Grade	Node	Glycoprotein 210	230	250	280
1	ID	2	pos	+FG	+F	–	–
2	ID	2	NK	+G	+F	–	–
3	ID	2	NK	–	–	+FG	–
4	ID	2	pos	+FG	+FG	–	+G
5	ID	2	NK	+F	+FG	–	–
6	ID	2	NK	–	+F	+FG	–
7	ID	2	pos	–	+F	–	–
8	ID	2	NK	+FG	–	+FG	+FG
9	ID	3	NK	+FG	+F	+FG	–
10	ID	3	pos	+FG	+FG	–	–
11	ID	3	pos	+G	+FG	+G	–
12	ID	3	NK	+FG	–	+G	+FG
13	ID	3	NK	+FG	–	+G	+FG
14	ID	3	pos	+FG	+F	–	+FG
15	ID	3	pos	+F	+G	–	+G
16	ID	3	neg	+FG	–	+FG	+F
17	Med	3	NK	+FG	–	+FG	+F
18	Secr	1	pos	ill defined high MW glycoproteins			

– = absent; + = present; G = galactose; F = fucose. ID = infiltrating duct carcinoma; Secr = secretory carcinoma; Med = medullary carcinoma. Node pos = metastasis present; Node neg = no evidence of metastasis; NK = node status not known. Grade 1 = well differentiated; 2 = moderately differentiated; 3 = poorly differentiated.

greater number of gps in the 210–280 kDa range identified in media from the carcinomas (Fig. 2) and these were frequently fucosylated (Figs. 2, 3 and 5, Table 2). The gp of MW 230 kDa was notable for not being detected from any of the benign samples but identified from 11 of 17 carcinomas, being fucosylated for 10 of these (Figs. 2, 3 and 5).

Differences were identified between the gps of less than 200 kDa from benign and malignant breast but these were generally quantitative or less consistent. The gp of MW 130 kDa was usually a more prominent band in the carcinoma samples (Figs. 2 and 3), whereas gp of 55 kDa was frequently more prominent in the benign cases (Figs. 3, 4–6). Several bands were readily identifiable for both benign and malignant specimens such as the gps of 110, 96, 64, 47, 35 and 18 kDa (Figs. 2–6).

Benign tissues

There was no significant difference in the number and MW size of the gps identified from the different cases, apart from the small number for which higher molecular weight gps were found. The latter appeared to relate to the degree of hyperplastic change (Table 1). Below 200kDa all gps identified showed incorporation of both [^{3}H]fucose and galactose (Fig. 4).

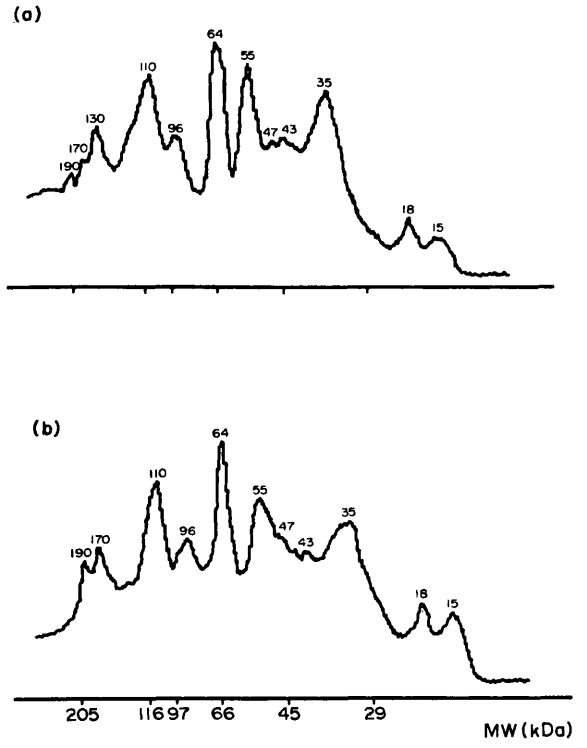


Fig. 4. Densitometric profiles of fluorograms of labelled glycoproteins released from benign mildly hyperplastic breast (case 7). 10–20% SDS-PAGE. (a) [³H]Galactose label. (b) [³H]Fucose label.

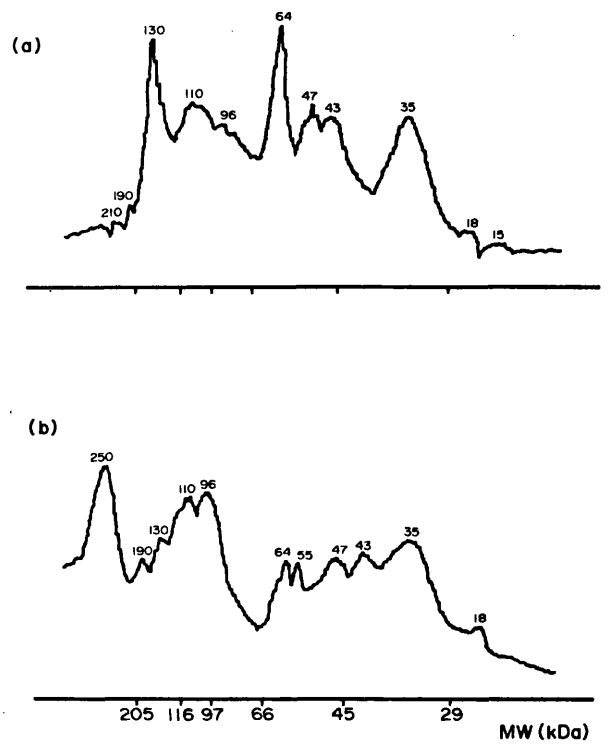


Fig. 6. Densitometric profile of fluorograms of [³H]galactose labelled glycoproteins. (a) Fibroadenoma (case 3). (b) Moderately differentiated carcinoma (case 6).

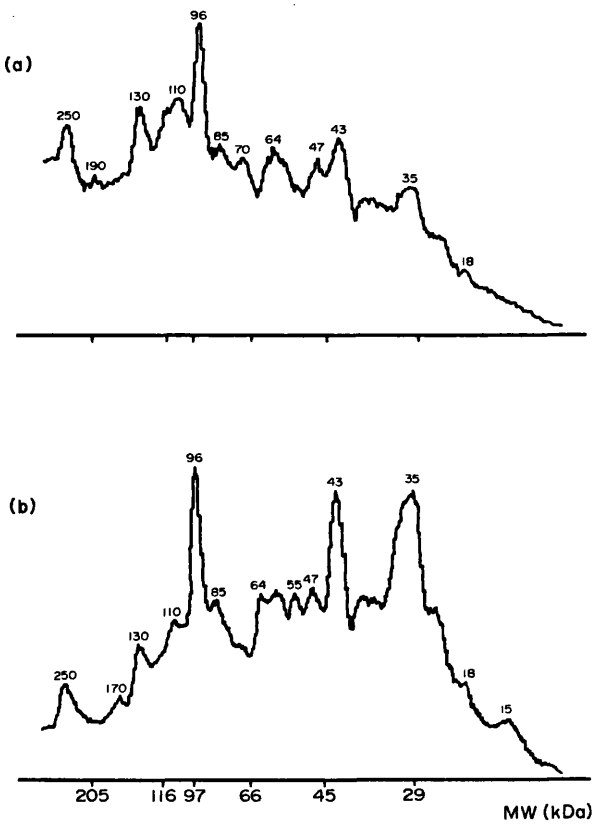


Fig. 5. Densitometric profile of fluorograms of labelled glycoproteins released from a moderately differentiated carcinoma (case 3). 10–20% SDS-PAGE. (a) [³H]Galactose label. (b) [³H]Fucose label.

Carcinomas

Adequate separation of the high molecular weight gps could not be achieved for one case, a secretory carcinoma. This was due to impaired migration from the stacking gel, which was probably related to the complex mixture of glycoproteins that are characteristic of this rare tumour.

An association was found between differentiation and the number of gps of MW 210–280 kDa identified, with 8/9 poorly differentiated carcinomas having three of the gps in comparison to 2/8 moderately differentiated tumours. Glycoproteins of 210, 250 and 280 kDa were more frequently identified from poorly differentiated carcinomas (gp210 9/9; gp250 6/9; gp280 6/9) than moderately differentiated tumours (gp210 5/8; gp250 3/8; gp280 2/8) whereas there was no difference for gp230. Insufficient information was available about lymph node status to draw any conclusions.

There was heterogeneity of fucosylation of the gps greater than 200 kDa (Table 2) and also in the MW region 40–80 kDa. An example of this is shown in Fig. 5 in which there are gps in this range which showed marked differences between the incorporation of [³H]fucose and [³H]galactose which was not seen in the benign samples (Fig. 4). However, there were carcinomas which behaved in the same way as the benign tissues.

Comparison of the lymph node metastases with their primary carcinomas showed that the patterns of gp release were similar, apart from heterogeneity of fucosylation of gps 50–55 kDa in one case. Histology confirmed that only metastatic carcinoma had been assessed.

DISCUSSION

This study has shown that there are significant differences in the nature of glycoproteins released from benign and malignant human breast *in vitro*, the main changes occurring with malignancy being related to glycoprotein size and fucosylation.

Increased expression of higher molecular weight glycoproteins occurring with malignancy has been reported for animal mammary tumours [18], including differences between rat mammary adenocarcinomas of low and high metastatic potential [19], but comparable studies on primary human tissues are limited. Gendler *et al.* [20] when examining the synthesis of [^{14}C]glucosamine-labelled glycoproteins by benign and malignant breast identified a group of acidic glycoproteins with molecular weights greater than 200 kDa which were more prominent in malignant specimens. In the present study there was a lower incidence of glycoproteins in the region 210–280 kDa from the benign tissues than the carcinomas. With the latter, the number of glycoproteins identified in this range increased with decreasing differentiation of the tumours, thereby stengthening the relationship of increased molecular weight size with malignancy.

Differences in lower molecular weight glycoproteins released from benign and malignant breast were also identified. Both increased (e.g. 130 kDa) and decreased expression (e.g. 55 kDa) of glycoproteins was found from tumours, whilst a number of bands were consistent between all samples. The band at 47 kDa could be the glycoprotein of MW 48 ± 6 kDa identified by Dermer and Tökes from benign [21] and malignant [22] breast, using similar methods. These findings indicate that the changes present in the higher molecular weight ranges were not related to technical variations. The similarities between the glycoproteins released from the 18 h and 48 h cultures for both benign and malignant tissues also suggest that the differences are not related to differences in cell surface turnover between the tissue types.

The higher (>200 kDa) molecular weight glycoproteins identified from benign and malignant breast also differed markedly in relation to the incorporation of [^{3}H]fucose. This was a feature of the glycoproteins released from carcinomas, although heterogeneity was prominent, but was essentially not found for the glycoproteins from benign breast. Heterogeneity of fucosylation of lower molecular weight glycoproteins (40–80 kDa) was also seen with carcinomas. Previous immunohistochemical studies have shown differences in expression of fucoproteins between benign and malignant breast, for example of the differentiation antigen 3-fucosyl-*N*-acetyllactosamine [23], which may reflect quantitative and/or qualitative differences in alpha-2- and alpha-3-fucosyl transferases or their regulation [24]. In studies of high- and low-metastatic cell lines [25–27] changes in fucose metabolism have been noted, which could have an influence on the biological behaviour of the cells.

The glycoprotein of MW 230 kDa was notable for being detected in 60% of carcinomas, when it was almost always fucosylated, but was not detected from any benign breasts. This was the only glycoprotein that showed such selectivity. Analysis by 2D electrophoresis, glycosidase digestion and peptide mapping is required to assess uniformity of structure between carcinomas. Similar methods of analysis need to be applied to the glycoproteins identified in this higher range, to assess similarities/differences between their protein and glycan structures, and also between those released from benign and malignant samples. Studies of those lower molecular weight glycoproteins which showed differences between benign tissues and carcinomas are also required. One of the major points of interest is the nature of the protein cores, since this would indicate whether the higher molecular weight glycoproteins released from carcinomas are formed completely *de novo* or whether they represent aberrant glycosylation of glycoproteins formed by normal breast. Data from analysis of mucins expressed by benign and malignant breast cells [28] indicates that the latter is more likely. Changes in the carbohydrate composition of glycoproteins involved in the function of normal cells could be of significance in determining the altered behaviour of malignant cells.

Glycoproteins synthesized by mammary cells of differing molecular weights have been recognized by many workers. A common approach has been by immunological analysis using monoclonal antibodies generated against breast tumours, cell lines and the milk fat globule membrane antigen. Of the higher molecular weight glycoproteins identified in this study, the one of 280 kDa may be the same or similar to that detected by the monoclonal antibodies generated by Major *et al.* [29]. Lower molecular weight glycoproteins recognized by antibody methods, e.g. of MW 43 kDa [30] and MW 19.5 kDa [31], may have been identified in the present study but did not exhibit the tumour specificity suggested by the previous reports.

As indicated, further work is required to investigate both the glycan and protein structure of these higher molecular weight glycoproteins, particularly gp 230 kDa. However it is anticipated that this will

provide an ideal immunogen for generating tumour-associated or even specific antibodies.

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Activation of the 'dense-vesicle' cyclic AMP–phosphodiesterase from rat liver by cyclic AMP-dependent protein kinase

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There are a number of distinct membrane-associated species of high-affinity cyclic AMP–phosphodiesterases in rat liver. However, only one of these enzymes, the so-called 'dense-vesicle' phosphodiesterase, can be activated by elevated intracellular cyclic AMP levels which ensue when hepatocytes are exposed to glucagon (Heyworth *et al.*, 1983). The effects of cyclic AMP on metabolic pathways are mediated by cyclic AMP-dependent protein kinase (hereafter designated A-kinase) which phosphorylates and hence modifies the activity of various enzymes (for review see Cohen, 1985). This study was undertaken to ascertain the effect of A-kinase on the 'dense-vesicle' phosphodiesterase from rat hepatocyte membrane fraction.

A crude membrane pellet prepared from rat hepatocytes was suspended in 20 mM-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose and various combinations of 5 mM-$MgCl_2$, 50 mM-sodium fluoride and 10 mM-β-glycerophosphate as indicated in Table 1. After preincubation at 30°C for 10 min, further additions were made such that all samples were adjusted to contain 50 mM-sodium fluoride, 10 mM-β-glycerophosphate and 5 mM-$MgCl_2$, and the incubations were continued at 30°C for a further 10 min with or without the catalytic subunit of A-kinase (100 unit/ml) and 0.1 mM-ATP as indicated in Table 1. Phosphodiesterase activity was then assayed as described by Marchmont & Houslay (1980), both in the presence and absence of 100 μM-ICI 118233.

Following preincubation of the hepatocyte particulate fraction with 5 mM-$MgCl_2$ for 10 min, subsequent incubation with A-kinase and ATP resulted in an increase in high-affinity cyclic AMP–phosphodiesterase activity (Table 1, experiment 2, $P<0.05$). The fraction of phosphodiesterase activity blocked by the compound ICI 118233 was used to assess the contribution of the 'dense-vesicle' phosphodiesterase to this activation of total phosphodiesterase activity as ICI 118233 has been shown to selectively inhibit the 'dense-vesicle' phosphodiesterase in liver (Pyne *et al.*, 1987*b*). We demonstrated that activation of the 'dense-vesicle' enzyme entirely accounted for the stimulation of phosphodiesterase activity which occurred during treatment with A-kinase (Table 1, experiment 2). Maximal stimulation of 'dense-vesicle' phosphodiesterase activity was achieved after incubation with A-kinase and ATP for 10 min. The magnitude of the stimulation of 'dense-vesicle' phosphodiesterase activity by A-kinase varied between preparations but was always between 2- and 3-fold. In the absence of ATP, however, A-kinase failed to stimulate phosphodiesterase activity (Table 1, experiment 4). When the particulate fraction was not preincubated without $MgCl_2$ the percentage stimulation of phosphodiesterase activity during subsequent incubation with A-kinase was markedly reduced and was insignificant (Table 1, experiment 1). When protein phosphatase inhibitors were included along with $MgCl_2$ during the preincubation, the ability of A-kinase to activate the 'dense-vesicle' enzyme was abolished (Table 1, experiment 3). Therefore, by activating protein phosphatases, $MgCl_2$ allowed A-kinase to activate the 'dense-vesicle' phosphodiesterase.

The results presented here show that A-kinase increased the activity of the 'dense-vesicle' phosphodiesterase in a particulate fraction prepared from rat hepatocytes. This only occurred when membranes were treated with Mg^{2+} before exposure to A-kinase and ATP. As such a pretreatment did not itself cause any activity change in the enzyme, we suggest that this phosphodiesterase may also be a substrate for a distinct kinase which serves not to regulate phosphodiesterase activity *per se*, but rather to attenuate the ability of A-kinase to activate this enzyme. Thus the 'dense-vesicle' phosphodiesterase may be subject to multisite phosphorylation.

A-kinase could have phosphorylated the 'dense-vesicle' enzyme directly or alternatively phosphorylated an intermediate kinase or phosphatase which acted upon the phosphodiesterase. the 'dense-vesicle' phosphodiesterase has been purified to homogeneity in our laboratory (Pyne *et al.*,

Table 1. *Effect of A-kinase on phosphodiesterase activity in a particulate fraction from rat hepatocytes*

The particulate fraction was preincubated at 30°C for 10 min with the additions indicated. Samples were then incubated for a further 10 min at 30°C with or without A-kinase (100 unit/ml) and 0.1 mM-ATP as indicated. Results are mean ± S.E.M.: *, ** indicate that the value is significantly different ($P<0.05$, $P<0.01$ respectively) from the value for the appropriate incubation in the absence of A-kinase. Abbreviations: BGP, β-glycerophosphate; PDE, phosphodiesterase; DVPDE, 'dense-vesicle' phosphodiesterase.

	Preincubation	Incubation		Total PDE	DVPDE
Experiment	Additions	ATP	A-kinase	(pmol/min per mg)	(pmol/min per mg)
1	—	+	+	3.0 ± 0.2	1.4 ± 0.1
	—	+	—	2.6 ± 0.1	1.2 ± 0. 1
2	$MgCl_2$	+	+	3.4 ± 0.3*	2.2 ± 0.2**
	$MgCl_2$	+	—	2.7 ± 0.1	1.0 ± 0.04
3	$MgCl_2$ + NaF + BGP	+	+	2.3 ± 0.2	1.0 ± 0.03
	$MgCl_2$ + NaF + BGP	+	—	2.5 ± 0.1	1.2 ± 0.1
4	$MgCl_2$	—	+	2.5 ± 0.1	1.0 ± 0.1
	$MgCl_2$	—	—	2.4 ± 0.1	1.0 ± 0.3

1987*a*) and further studies will be carried out with such a pure preparation to establish whether or not A-kinase can directly phosphorylate the 'dense-vesicle' enzyme.

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Altered expression *in vitro* of human breast tumour associated glycoproteins by hormone additives

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Modifications in the glycosylation of cell surface glycoproteins are known to be linked with malignant transformation (Warren & Buck, 1980). Modifications or altered expression of these glycoproteins could be of interest in the determination of tumour cell behaviour, since cell surface glycoconjugates have been implicated in many cell–cell interactions (Olden *et al.*, 1982). Altered glycoproteins may be of value clinically as tumour-associated markers.

Recent studies have shown the presence of several high molecular mass glycoproteins in human breast tissue, which appear to be associated with malignancy (Walker & Rye, 1987). These glycoproteins, of M_r 210 000–280 000 released into media from organ cultures, have a considerably higher incidence in poorly differentiated carcinomas than in benign breast. A fucosylated glycoprotein of M_r 230 000 was of particular note, since it was detected in 11 of 17 carcinomas, but in none of 10 benign samples tested.

There has been increasing interest in the concept of modulation of phenotypic expression of antigens in breast carcinomas (McGuire *et al.*, 1985). To date, the effects of interferon on tumour-associated antigen expression has been assessed by fluorescence activated cell sorting (FACS) analysis (Greiner *et al.*, 1984), and the effects of insulin and hydrocortisone on breast antigens by immunohistology (Jones & Walker, 1987). The latter study showed that hydrocortisone has a possible proliferative effect on breast cancers in culture, while insulin has an effect on the antigen expression of certain carcinomas, resulting in better functional differentiation.

In the present investigation, a different, biochemical approach for assessment of modulation has been used. The expression of glycoproteins of M_r 210 000–280 000 by breast carcinomas *in vitro* was assessed in the presence or absence of the corticosteroid hormone hydrocortisone and the peptide hormone insulin. Receptors for both of these hormones have been detected in a substantial proportion of breast carcinoma specimens (Fazekas & MacFarlane, 1977; Holdaway & Friesen, 1977).

Six primary breast carcinomas were cultured in Dulbecco's modified Eagle's medium with L-glutamine, using the raft method of Wellings & Jentoft (1972), in an atmosphere of air/CO_2 (19:1) at 37°C. All tissues were incubated with [^{3}H]fucose (20 μCi/ml) with either no hormone additives (control), insulin (5.0 μg/ml) or hydrocortisone (1 μg/ml). After 48 h, the media was dialysed, concentrated and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Densitometry of the fluorographs for four of the carcinomas showed that organ culture in the presence of insulin led to a specific decrease in the detection of the high molecular mass glycoproteins, most notably the M_r 230 000 glycoprotein. Parallel incubations with hydrocortisone showed a marked increase of the glycoproteins of M_r 250 000 and 230 000 plus one of M_r 110 000 when compared with the control tissues (Fig. 1). In the two remaining samples analysed, little effect was observed with the addition of either hormone. Viability was satisfactory in all samples. The responsiveness of the carcinomas appeared to relate to their histological differentiation, in that only the moderate to poorly differentiated tumours (grades II and III) showed modulation with the addition of either hydrocortisone or insulin to the culture medium.

These preliminary findings are of interest with regard to the nature of the glycoproteins in the range M_r 210 000–280 000 and their relationship to tumour differentiation. Insulin has previously been shown to result in enhanced expression of differentiation related markers and suppression of tumour-associated antigens in a small group of breast carcinomas *in vitro*, using less sensitive methods (Jones & Walker, 1987). The present study indicates that there is suppression of specific glycoproteins by insulin which could either be due to an effect on the biosynthesis of the glycoproteins or an alteration in their release from carcinomas. The findings support the previous evidence that the higher molecular mass fucosylated glycoproteins, in

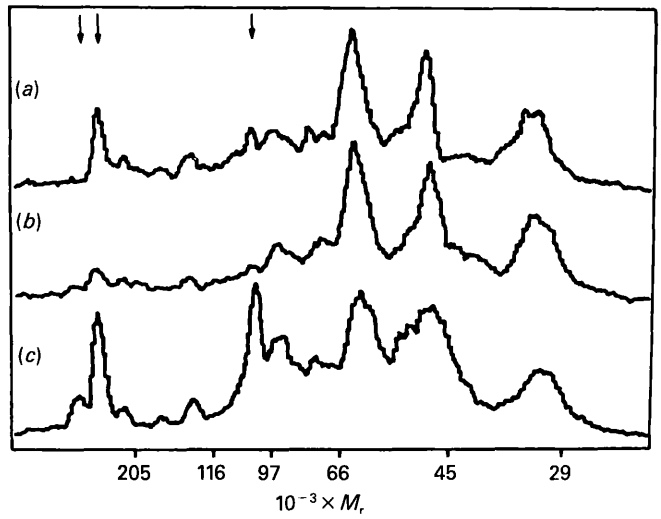

Fig. 1. *Densitometry traces of [^{3}H]fucose-labelled glycoproteins released from organ culture of primary breast carcinoma*

(*a*) After incubation without hormones, (*b*) with insulin (5.0 μg/ml) and (*c*) with hydrocortisone (1.0 μg/ml). Arrowed peaks (from left) refer to the glycoproteins of M_r 250 000, 230 000 and 110 000.

particular that of M_r 230 000, are tumour associated and may therefore be of clinical value.

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Receptor-mediated endocytosis by human Kupffer cells

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Previous studies using experimental animal systems have shown that the liver is the primary site of metabolism of circulatory carcinoembryonic antigen (CEA). Hepatic metabolism of CEA is a multistep process involving both Kupffer cells and hepatocytes (Toth *et al.*, 1982). CEA is removed from the circulation by a receptor on the Kupffer cell specific for CEA and non-specific cross-reacting antigen (NCA), a structurally related glycoprotein (Toth *et al.*, 1985; Thompson *et al.*, 1987). Clinical evidence of elevated CEA levels in patients with benign liver disease has also implicated the liver as the site of CEA metabolism in the human (Thomas & Zamcheck, 1983). To determine if the mechanism in humans is similar to that in experimental animal systems, we isolated Kupffer cells from fresh human liver.

Normal liver was obtained from patients undergoing lobectomies for isolated hepatic metastases. The liver was sliced thinly and rinsed in phosphate-buffered saline to remove as much blood as possible. The liver slices were minced and enzymically digested by incubation in 0.05% (w/v) collagenase buffer at 37°C for 40 min with agitation. The liver digest was strained, pelleted and resuspended in fresh collagenase buffer and incubated for an additional 20 min. Cell clumps and hepatocytes were removed from the resulting cell suspension by repeated low speed centrifugation (50 ***g***, 2 min). The resulting single-cell suspension was washed several times and centrifuged in a 17.5% (w/v) metrizamide gradient (1400 ***g***, 15 min) at room temperature. The Kupffer cell fraction was harvested from the top of the gradient and final purification was achieved by adherence to tissue culture dishes at 37°C for 8 h. The resulting cells were greater than 95% viable by Trypan Blue dye exclusion and contained less than 1% hepatocytes. The Kupffer cells were identified by staining for non-specific esterase and peroxidase activity, phagocytosis of colloidal carbon and 0.8 μm latex beads, and by electron microscopy (Fig. 1). The isolated cell population was composed of 60–70% Kupffer cells after the metrizamide gradient and 80–85% Kupffer cells after the final adherence step.

The isolated cells took up both radiolabelled ^{125}I-CEA and yeast mannan in a time-dependent manner. The uptake of CEA was concentration dependent and saturable. Endocytosis of radiolabelled CEA was inhibited by 67% in the presence of a 400-fold molar excess of unlabelled CEA. NCA is also recognized by the Kupffer cell receptor in the rat, and endocytosis of CEA by the human Kupffer cells was inhibited by 68% in the presence of a 1000-fold molar excess of NCA.

Abbreviations used: CEA, carcinoembryonic antigen; NCA, non-specific cross-reacting antigen.

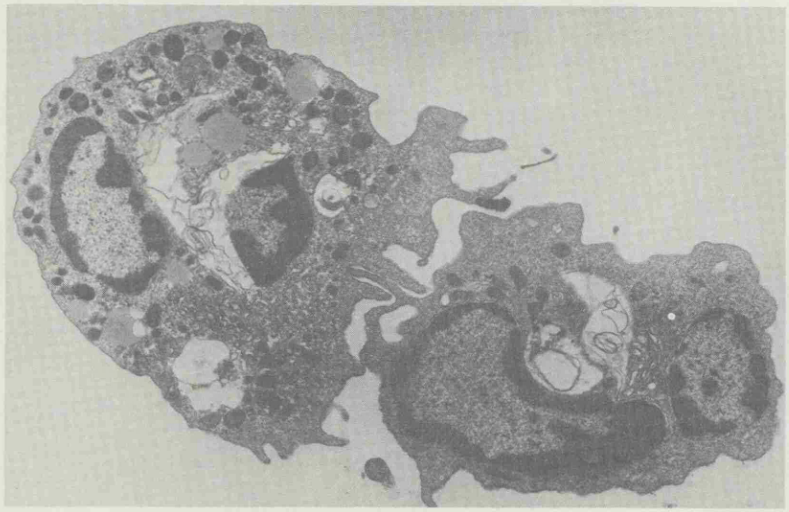

Fig. 1. *Electron micrograph of two isolated human Kupffer cells*
Magnification: × 6000.

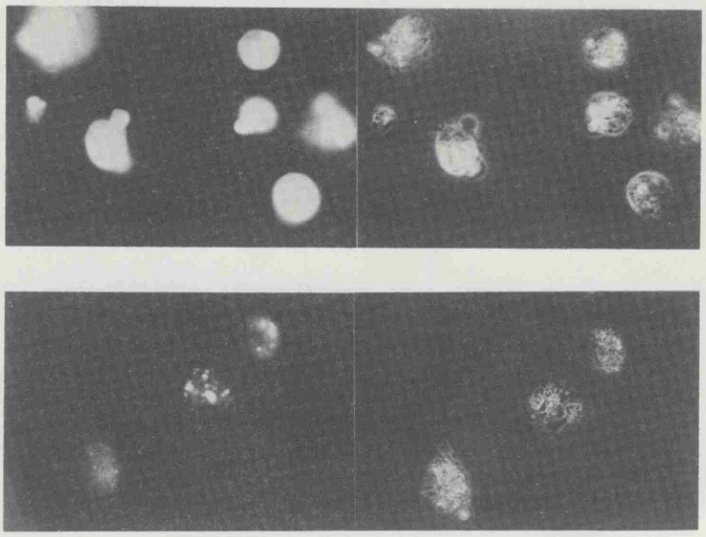

Fig. 2. *Binding of CEA conjugate inhibited by excess unlabelled CEA*

Top: binding of rhodamine-conjugated CEA (10 μg/ml) to isolated human Kupffer cells ($\times$430). Bottom: inhibition of rhodamine-conjugated CEA (10 μg/ml) binding to human Kupffer cells in the presence of unlabelled CEA (1 mg/ml). Left: fluorescence at 640 nm. Right: phase contrast.

Isolated human Kupffer cells bound and internalized rhodamine–isothiocyanate-conjugated CEA as assessed by fluorescence microscopy. The binding of the CEA conjugate was specific and could be inhibited by an excess of unlabelled CEA (Fig. 2). In addition, we were also able to show specific binding of CEA to the sinusoidal areas of snap-frozen human liver by immunofluorescent staining using rhodamine-conjugated CEA. However, it was not possible to distinguish Kupffer cells from other sinusoidal cells in the liver sections.

Conclusions

CEA is taken up by human Kupffer cells in a saturable and concentration-dependent manner. The receptor also recognizes NCA. The kinetics of cellular uptake were similar to those previously reported for rat Kupffer cells. It is likely that the mechanism of circulatory clearance of CEA in the human is similar to that in the rat.

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A model of Ca^{2+}–heparin interaction

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Heparins are strongly anionic, and interact with a variety of cationic species. Many possible modulations by metal ions, including Ca^{2+}, of the activities *in vivo* and *in vitro* of heparin (and of heparans, which contain less sulphate groups and iduronic acid residues, and more acetate groups and glucuronic acid residues than heparin) have been discussed (e.g. Long & Williamson, 1979, 1982). The molecular mechanisms underlying heparin–Ca^{2+} interactions are not clear, but are likely to be complex. An equilibrium dialysis study suggested a biphasic reaction (Grant *et al.*, 1986*a*). Analyses by n.m.r. demonstrated that Ca^{2+} binds predominantly to the carboxyl groups of heparin, with the 2-sulphamino groups playing a subsidiary, but essential, role (Liang & Chakrabarti, 1982; Ayotte & Perlin, 1986). It has been suggested, as a result of an analysis of the patterns of heparin and heparan 2-sulphamino group i.r. absorptions, that in the presence of Ca^{2+} (and also under some other conditions), the glycosaminoglycan chain is held in a conformation that is stabilized by an interaction between the 2-sulphamino and carboxylate groups, possibly involving hydrogen bonding (Grant *et al.*, 1987*a*,*b*). An additional complication involves the probable involvement of co-ordinated water molecules in at least some heparin–metal ion interactions (Grant *et al.*, 1986*b*). In assessing the biological relevance of such interactions, the chemical heterogeneity of the polysaccharide chains must be borne in mind. Thus, the cation interaction in parts of heparin bearing 3-*O*-sulphated glucosamine residues (i.e. high-affinity antithrombin-binding sites) seems to be different from that occurring elsewhere in the polymer (Grant *et al.*, 1987*c*). In the present communication, a study by ^{13}C-n.m.r. spectroscopy and by pH titration of Ca heparinate is interpreted in terms of a stereochemical model of Ca^{2+}-heparin interaction.