Glycoproteins: Biosynthesis and Location in the Nuclear Membranes of BHK Cell Lines

by

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SUMMARY

The nuclear membrane glycoproteins of BHK cells were studied in several ways.

Hydroxyurea and cytosine arabinoside are inhibitors of DNA synthesis and of $[^3H]$-glucosamine incorporation into intracellular glycoproteins. These two inhibitors with a third, 2-deoxy-D-glucose, were used as a potential means of elucidating the steps by which membrane glycoproteins are transported and secreted extracellularly.

2-Deoxyglucose proved a powerful inhibitor of $[^3H]$-glucosamine uptake by BHK cells after only a short period of incubation, while allowing protein synthesis to occur at a reduced rate. 2-Deoxyglucose was found to exert no influence on the secretion of acid precipitable $[^3H]$-glucosamine into the growth medium. The nature of the labelled extracellular glycosylated material was found to be, glycoprotein hyaluronic acid and choindroitin sulphate.

In order to study the glycoproteins of the nuclear membrane, the isolation of nuclei, uncontaminated by other cellular components was necessary. A method of preparation is described which effects this without removal of the nuclear membrane.

The possibility of preferential extraction of the glycoproteins from nuclear membrane was investigated using non-ionic detergents, although these proved to be non-selective in their solubility effects.

The nuclear membrane from $[^3H]$-glucosamine labelled BHK C13 cells and from their polyoma transformed counterparts (PYY), was isolated by two methods. One method involved the polyanion, heparin; the other was sonication. Recovery of the $[^3H]$-glucosamine label was higher using the heparin method.

Two bands of nuclear membrane material were consistently isolated on a sucrose step gradient at the 1.22/1.20 g ml^{-1} and 1.20/1.18 g ml^{-1}

sucrose interface.

In addition, a high proportion of the crude nuclear membrane material applied to the sucrose density gradient remained at the top of the gradient.

Endoplasmic reticulum was isolated on an identical sucrose step gradient. Most of the material was found at the top of the gradient and in the pelletted material. It is suggested that the detergent Tween 80 removed endoplasmic reticulum contaminated nuclei.

Citric acid treatment of nuclei is reported to remove the outer nuclear membrane. A nuclear membrane preparation from citrate-washed BHK nuclei predominantly reduced the level of $[^3H]$-glucosamine at the 1.20/1.18 g ml^{-1} interface, suggesting that this fraction was enriched in the outer nuclear membrane.

The final nature of the $[^3H]$-glucosamine label introduced to the cell growth medium was found in the nuclear membrane - as glucosamine, galactosamine (not in PYY nuclear membrane) and sialic acid.

Nuclear membrane glycoprotein components isolated from BHK cell lines were shown to have a binding affinity for the (lentil) lectin, Lens culinaris when the latter was coupled to Sepharose 4B. In this respect PYY membrane Band 2 components behaved anomalously in that a high proportion of the binding to the lectin was irreversible. Differences in the glycoprotein SDS polyacrylamide gel profile of nuclear membranes from both C13 and PYY were observed.

INTRODUCTION

1.1. General

In recent years it has been increasingly recognised that small amounts of carbohydrate (1-10% $^{w}/_{w}$) (for reviews see Cook & Stoddart, 1973; Hughes, 1973) are associated with cellular membranes. Furthermore, it is well established that malignant cells possess modifications of this membrane bound carbohydrate, compared with normal cells. These changes in the oligosaccharide structure have been demonstrated to be present both in glycolipids (Hakomori, 1975) and glycoproteins (Warren et al., 1974).

Glyco-substances of intracellular membranes have been detected particularly in mitochondrial membranes (Bosmann et al., 1972; Nicolson et al., 1972) and on nuclear membranes (Nicolson et al., 1972; Sieber-Blum & Burger, 1977). It would therefore be of interest to know if the biosynthesis of intracellular membrane glycosylated protein is changed upon transformation of a cell, in common with plasma membranes.

The purpose of this study was to isolate the nuclear membrane of Cl3 baby hamster kidney (BHK) cells and subsequently to determine if glycoproteins exist therein; to define them structurally and further to compare them with those isolated from their polyoma transformed counterparts (PYY).

1.2. Membrane Glycoproteins -

1.2.1. Structure

There are two major classes of glycoprotein. Those that are secreted from cells and those that may be classified as membrane constituents. A resumé of structure and function will be confined to the latter.

A glycoprotein, by definition, is a polypeptide chain having oligosaccharide side chains covalently bound to it. The glycophorin molecule (the major sialo-glycoprotein constituent of the erythrocyte membrane) is the only membrane glycoprotein which is structurally well defined. This is an asymmetric molecule in which the carbohydrate residues are attached extracellularly at the N-terminal end of the peptide chain (Moraweicki, 1964). The glycoprotein spans the entire membrane (Bretscher, 1971a & b; Boxer et al., 1974; Mueller & Morrison, 1974) and the protein backbone has been sequenced from tryptic and CNBr studies (Marchesi et al., 1972; Jackson et al., 1973; Segrest et al., 1973; Marchesi et al., 1976) as have the oligosaccharides for some receptors e.g. the phytohaemagglutinin receptor site (Kornfeld & Kornfeld, 1970).

A small group of sugars plays a key role in the structure of glycoproteins. The multiplicity of bonding which may occur between the different sugar residues leads to oligosaccharide sequences of great heterogeneity - an important contributory factor in the recognition role of cell surface glycoproteins. Three types of sugars make up the oligosaccharide backbone:-

i) the neutral sugars:- D-galactose, D-mannose, L-fucose.

ii) the amino sugars:- D-galactosamine, D-glucosamine, which are normally N-acetylated.

iii) the sialic acids, which may be N- and o-acetylated or have N-glycolyl substituents.

The sugar chains may be linked to the protein backbone in two possible ways, either by a 'serum' type linkage - an alkali stable β-linkage between N-acetylglucosamine and an asparagine residue of the peptide; or by a 'mucin' type linkage - an alkali labile glycosidic α-linkage between N-acetylgalactosamine and serine or threonine. The permutations possible as a result of the admissable alternations would appear to be restricted by the following set of rules:-

1. Only specific sugars are linked to the peptide chain.
2. Mannose, N-acetylglucosamine and; N-acetylgalactosamine are the core sugars.
3. Fucose and sialic acid are terminal sugars i.e. found at the non-reducing end of the saccharide.
4. Galactose usually precedes N-acetylneuraminic acid at the non-reducing end or alternatively is found along with N-acetyl-glucosamine subterminal to fucose.

Microheterogeneity is a property of glycoproteins which may have some structural significance. This describes the situation whereby the oligosaccharide units attached to the peptide are at varying stages of completion or where alternative pathways of synthesis are possible. A consideration of the roles which glycoproteins play in vivo will help to exemplify the importance of the carbohydrate moiety of the molecule.

1.2.2. Function of Membrane Glycoproteins

1.2.2.1. Role in Adhesion

Roseman (1970) suggested that glycosyl transferases located on the cell surfaces played a part in cellular adhesion by interacting with glycoprotein/glycolipid acceptors in other cells, forming an enzyme-substrate complex with a finite half-life. The main criterion in considering the Roseman Hypothesis is whether or not such enzymes exist on the cell surface at all. There is evidence, however, (Jamieson

et al., 1971; Bosmann, 1971) that cell surface glycosyl transferases are involved in platelet-collagen adhesion occurring during blood clotting. In addition, such transferases have been demonstrated to be present on the outer surfaces of chick embryo cells and neural retinal cells (Roth et al., 1971), rat intestinal epithelial cells (Weiser, 1973) and mouse fibroblast cells (Roth et al., 1974).

If acceptance of this theory is assumed, then the number of possible donor carbohydrate structures of surface glycoproteins and the precise acceptor requirements of the glycosyl transferases mean that the resulting adhesion could be a mechanism of extremely high specificity.

1.2.2.2. Role in Antigenic Properties of Cell Surface Carbohydrates

All cells induce an immune response if injected into a genetically dissimilar animal. This in itself suggests that cells differ structurally at their surfaces. It is known from long-standing studies on blood group substances that complex carbohydrates play a key role in defining the structural specificity of cell surfaces e.g. the predominant sugar which confers specificity in blood group A (human) is N-acetylgalactosamine; while in blood group B, galactose is the main sugar responsible for specificity (Watkins, 1972). These carbohydrates which are responsible for such properties are present in the red cell glycolipids. The glycoproteins of the rbc membrane are not known to carry the A, B, H antigenic determinants, although a glycophorin oligosaccharide comprising, sialic acid, galactose and N-acetylgalactosamine is thought to be responsible for MN antigenicity (Thomas & Winzler, 1969a & b) - the second major human blood group system. The presence of sialic acid residues appear to be significant, since the M and N antigenicity is destroyed by treatment with neuraminidase.

1.2.2.3. Recognition and Clearance of Asialoglycoproteins: Role of Sialic Acid

The role of carbohydrates in determining the survival of serum glycoproteins has been brought to light by a series of experiments by Morell and co-workers (Morell et al., 1968; Van den Hamer et al., 1970; Morell et al., 1971). The enzymatic removal of terminal sialic acid from ceruloplasmin (a serum glycoprotein) results in its rapid clearance from the circulation. Further, removal of sub-terminal galactose allowed ceruloplasmin to circulate normally. The desialysed glycoprotein was found to be taken up by the parenchymal cells of the liver. These cells were presumed to have at their surface receptors specific for galactose.

Rogers and Kornfeld subsequently showed (1971) by coupling the glycopeptides of fetuin and to a lesser extent γ-G-immunoglobulin to a test molecule (lysozyme or albumin) and treating with neuraminidase that the resulting asialoglycopeptide of fetuin coupled to lysozyme/albumin is selectively cleared from the circulation by the liver.

The sialic acid content of red blood cell membranes may also be an important factor in the process whereby these cells are cleared from the circulation by the spleen (Jancik & Schauer, 1974). If sialic acid residues are removed, rapid clearance follows, indicating perhaps some role of sialic acid in the ageing process of red blood cells.

Yet another role of terminal sialic acid and sub-terminal β-galactoside has been demonstrated by Sanford (1967):- that neuraminidase treatment can alter the transplantation behaviour of a tumour. The TA3 tumour is characterised by a cell surface coating, a heavy sialomucin layer, demonstrable by histochemical staining. When neuraminidase treated TA3 cells are injected into C3M host (mouse) cells the ability of the liver to grow was markedly decreased (although not

in all allogenic host cells). Sialic acid is a component of the sialomucin layer (Gottschalk, 1956). Sanford suggests that it is possible, therefore, that its removal disrupts a protective cell surface coating which previously concealed cryptic tumour cell surface antigens from host defences.

Fucose, the other terminal saccharide of glycoproteins has also been associated with the recognition of lymphocyte secreted macrophage inhibition factor by macrophages in vitro (Remold, 1973).

A consideration of the function of glycoproteins in vivo gives some indication of the roles played by terminal and sub-terminal carbohydrate residues. The role of the core sugars is not clear.

1.2.2.4. Carbohydrate Structure and Surface Receptors

One aspect of glycoprotein chemistry in which there has been recent interest is that they can act as receptors on the cell surface for substances (e.g. hormones) inducing a biological response in target cells. The chemistry of these surface receptors for various hormones is the subject of a recent review by Cuatrecasas,(1974).

Because the carbohydrate moiety of the glycoprotein molecule may influence cell growth and play a part in cell-cell interactions, it may therefore also be important in malignancy. Investigations to date in this field have been hampered by the fact that there are no available methods for the removal of sugar residues, other than those of sialic acid, from proteins in a non-destructive way.

Glycoprotein involvement in changes which normal cells undergo after transformation are discussed in another section.

1.2.3. Glycoprotein Biosynthesis

Early experiments to elucidate the biosynthetic pathway of glycoproteins consisted of injecting whole animals with radioactive precursors, such as amino acids or sugars and investigating the fate of the label. More recently radioactive precursors have been incor-

porated into tissue culture cells, although it is possible that the culture media, which normally contains a high level of glucose, can affect the level of incorporation of a labelled sugar, such as glucosamine. In addition, that serum of the media also contains free sugars such as free glucosamine and steps may have to be taken to avoid dilution of the precursor e.g. dialysis of the serum (Hughes & Clark, 1974).

Labelled glucosamine is a particularly useful tracer for biosynthetic experiments because it permits the full glycosylation procedure to be monitored. Glucosamine, being the sugar by which the carbohydrate moiety is attached to the protein, is useful for studying the initial stages of glycosylation. It is a core sugar and therefore can provide information about middle stages; and label from glucosamine also appears in sialic acid (Fig. 1), which is a terminal residue.

The biosynthesis of glycoproteins can be controlled at three key stages. These being the points of

1.2.3.1. sugar phosphorylation and interconversion (Fig. 1)

1.2.3.2. sugar activation

1.2.3.3. sugar transfer to the peptide backbone.

1.2.3.1. Sugar Phosphorylation and Interconversion

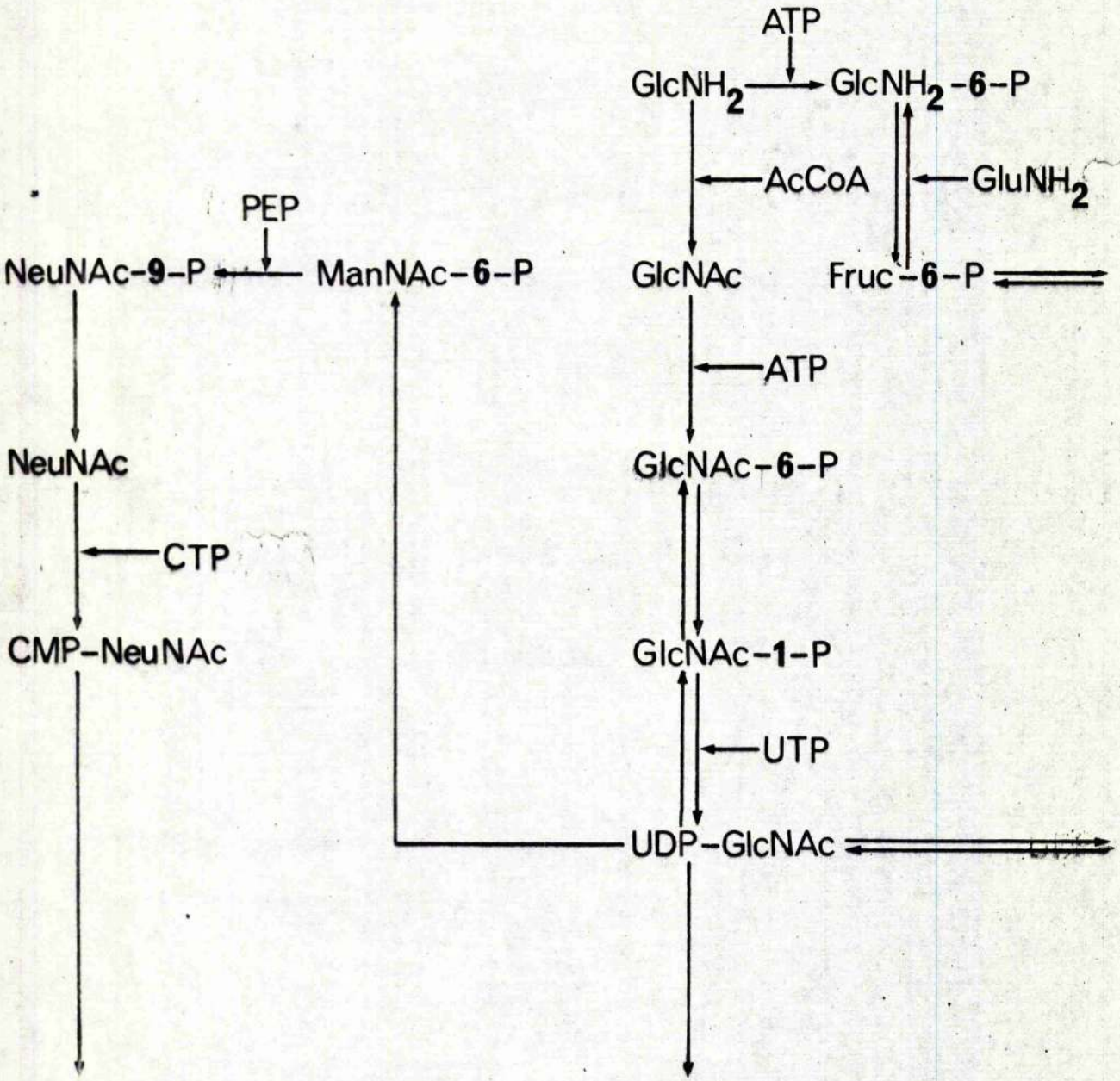
Fig. 1 shows the possible interconversions of the key sugar glucosamine and highlights its central importance in glycoprotein metabolism.

1.2.3.2. Sugar Activation

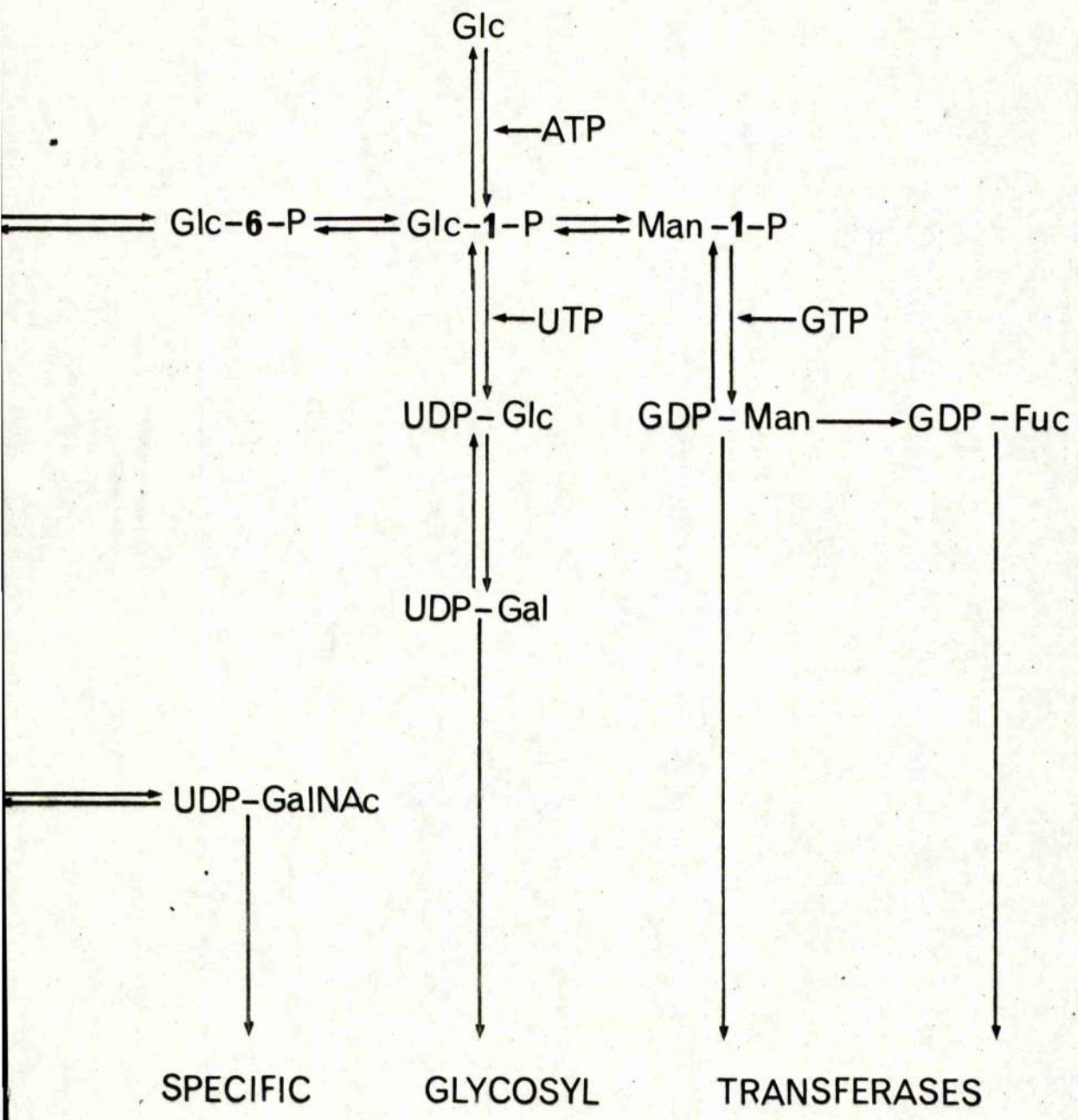
Oligosaccharide synthesis on the polypeptide backbone requires activated sugars in the form of sugar nucleotides. For most sugars these molecules are synthesised in the cytoplasm by nucleoside-5'-diphosphate sugar synthetases i.e. as sugar diphosphonucleotides.

Sialic acid in the activated form is cytidine-mono-phosphate-N-acetylneuraminic acid (CMP-NANA). The synthetase catalysing the

Fig.1 Glucosamine in Glycoprotein Biosynthesis



GLYCOSYLATION OF POLYPEPTIDES BY


Glc
ATP
Glc-6-P
Glc-1-P
Man-1-P
UTP
GTP
UDP-Glc
GDP-Man
GDP-Fuc
UDP-Gal
UDP-GalNAc
SPECIFIC
GLYCOSYL
TRANSFERASES

activation of NANA is found exclusively in the nucleoplasm - not the membrane, since the enzyme is unaffected by the Triton X100 (Van Dijk, 1973). Since sialation of the glycopeptide is an extra-nuclear event, it seems likely that the nuclear membrane must be exerting some control, either in the entry of N-acetylneuraminic acid into the nucleus to form the nucleotide sugar, or on the exit of CMP-NANA for sialation events at the golgi apparatus.

1.2.3.3. Sugar Transfer to the Peptide Backbone

It has been found from amino acid labelling experiments (Hallinan et al., 1968; Priestley et al., 1969) that the polypeptide moieties of glycoproteins are synthesised on the membrane bound ribosomes of the rough endoplasmic reticulum (ER). Radioactive pulse chase experiments indicate that there is a subsequent clear migration of label from the rough ER into the smooth ER and the golgi apparatus, the attachment being catalysed at each site by a different series of glycosyl transferases. Since labelled sugars are rapidly incorporated into the smooth ER and golgi, it is considered that these are the sites of attachment of the saccharides to the peptide. It would appear that the position of a particular saccharide residue on the peptide backbone is related to the site at which the sugar is added e.g. terminal residues like sialic acid are added at a later stage in the golgi.

To attempt to understand the mechanism of these biosynthetic events inhibitors of glycoprotein synthesis such as 2-Deoxyglucose may prove to be useful tools of investigation.

1.2.4. Inhibitor Studies

A second approach to this investigation involved the use of inhibitors as a means of elucidating the pathway of glycoprotein synthesis and extracellular transport.

1.2.4.1. Hydroxyurea and Cytosine Arabinoside

These two inhibitors have been reported to inhibit DNA replication in bacterial and animal cells (Roy-Burman, 1970; Graham and Whitmore, 1970). The 5'-triphosphate derivative (ara CTP) of 1-β-D-arabinofuranosylcytosine (ara C) specifically inhibits the enzyme DNA polymerase in bacterial cell extracts (Rama Reddy et al., 1971). Hydroxyurea inhibits DNA replication by blocking the action of ribonucleotide diphosphate reductase (Young et al., 1967).

However, ara C and its derivatives and also hydroxyurea inhibit directly the incorporation of $[^3H]$-glucosamine into the glycoproteins of hamster embryo fibroblasts and transformed cells (Hawtrey et al., 1973).

It is known (Bosmann, 1972; Graham and Whitmore, 1970 a) & b); Weiser, 1973 a) & b); Van Dijk et al., 1973) that radioactively labelled sugars are transferred by glycosyl transferases from their activated nucleotide carriers to glycosylated polypeptides which subsequently appear at the cell surface. Hawtrey et al. (1974), investigating the site of action of ara C derivatives and hydroxyurea, concluded that these inhibitors directly interfere with the process of saccharide transfer and, furthermore, that glycoprotein synthesis is also inhibited as a direct interaction between ara-CTP and CMP-N-acylneuraminic acid synthetase which actives N-acetylneuraminic acid.

1.2.4.2. 2-Deoxyglucose

The compound 2-deoxyglucose has also been widely used to inhibit the synthesis and secretion of glycoproteins. Evidence for this comes from the work of Farkas et al. (1970) on the cell wall proteins of yeast protoplasts. It was found that 2-deoxyglucose had a negligible effect on protein synthesis, but did exert a considerable inhibitory influence on carbohydrate and protein secretion into the medium. The

mechanism for this inhibition was considered as an interference of the metabolites of 2-deoxyglucose (viz. GDP-2-deoxyglucose) with the synthesis of yeast mannan.

In 1972, Ghandi et al., concluded from experiments using Hela cells infected with influenza virus that 2-deoxyglucose and glucosamine inhibit the synthesis of glycoproteins (e.g. the neuraminidase present on the viral coat). Those viral proteins lacking carbohydrate were unaffected by the inhibitor. The mechanism of this action is unclear, but the following explanation was ventured: Glucosamine, being intracellularly converted to its N-acetyl derivatives or N-acetyl phosphate derivatives, leaves a marked decrease in the uridine nucleotide pools as UDP-N-acetylgalactosamine in the activated form. Deoxyglucose becomes phosphorylated by ATP as soon as it enters the cell, thus reducing the intracellular adenine nucleotide pools. Glycoprotein synthesis is inhibited by creating an imbalance in the nucleotide pools of the cell required for sugar activation necessary for subsequent glycosylation of glycoproteins.

Further to this, Melchers (1973) reported that 2-deoxyglucose, at a maximum concentration of 3 mg ml^{-1}, inhibits the glycosylation of the immunoglobulin G1 in tumour plasma cells. Although the precise mechanism of this inhibition is as yet unknown, Melchers specifies the site of inhibitory action as being at the points of a) migration of the newly synthesised IgG1 polypeptide from the membrane-bound polyribosomes to the cisternae of the rough endoplasmic reticulum; and b) at the transfer of the IgG1 molecules from the rough to the smooth endoplasmic reticulum.

1.2.4.3. Tunicamycin

A recent paper (Schwartz et al., 1976) suggests that the new glucosamine-containing antibiotic tunicamycin (from Streptomyces

lysosuperificus) unlike the other inhibitors specifically inhibits glycosylation without influencing other metabolic reactions.

The effect of tunicamycin on the uptake and incorporation of labelled sugars into Semlike forest virus (SFV) infected cells was monitored by Schwartz and coworkers. Radioactive mannose in tunicamycin treated cells was reported to be decreased by 40%, while incorporation into the TCA-precipitable material was inhibited by 95%. These authors also reported that while general incorporation of glucosamine in SFV-infected cells remains unaffected, the glycosylation of SFV-specific glycoproteins with glucosamine is almost completely suppressed by tunicamycin.

Such an inhibitor could prove to be useful in future biosynthetic studies.

1.3. Membranes

Before considering intracellular systems, some current concepts of cellular membranes in general will be reviewed.

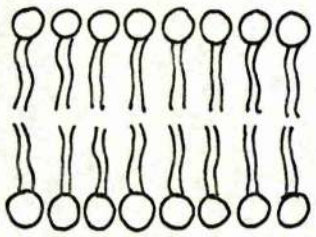
1.3.1. General Features

A membrane is not an inert, static structure, but a dynamic system in which the components are in a constant state of flux. A membrane provides a cell with a boundary which is selectively permeable to its environment, controlling the exchange of ions and metabolites; and allowing for the transport of particulate matter (endo- and exocytosis) between the cell and its surroundings.

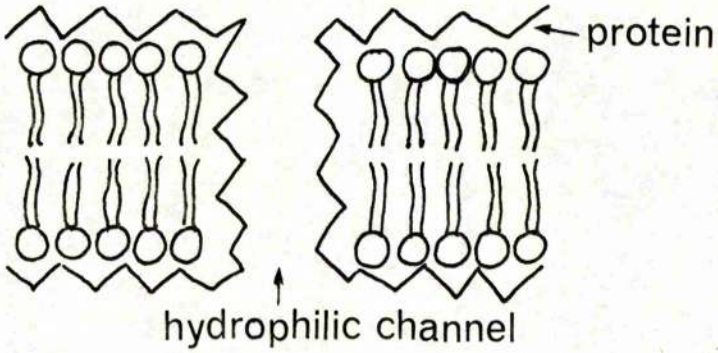
Early membrane studies were carried out on the red blood cell which provided a simple, readily available system. The work of Langmuir (1917), Gorter & Grendel (1925) and Danielli & Davson (1934) provided a basic membrane model of a lipid bilayer with associated protein. More recent investigations into the nature of this association has not detracted from the validity of this basic membrane model - a bimolecular leaflet with an internal hydrophobic region and an external hydrophilic region. The essential features of this model have been incorporated into the Fluid Mosaic Model of Singer & Nicolson (1972). They propose a discontinuous lipid bilayer in which the lateral movement of the lipid molecules is rapid (Kornberg & McConnell, 1971), but migration between monolayers ("flip-flop") is a rare event (Kornberg & McConnell, 1971). The outstanding features of this system as opposed to all the other classical models is that it emphasises the dynamic nature of the membrane and it distinguishes between two types of membrane protein - the "integral" proteins or "intrinsic" (Capaldi & Green, 1972) and the "peripheral" or "extrinsic" (Capaldi & Green, 1972). Integral globular proteins (some of which are glycoproteins) are amphipathic and embedded, in some cases even spanning the bilayer (Bretscher, 1971a & b). The

Fig. **2**

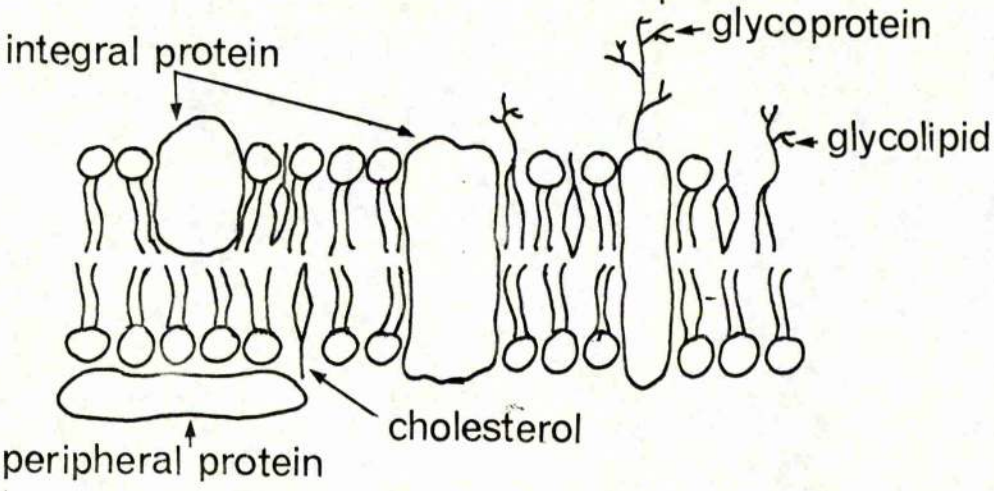
Gorter & Grendel lipid bilayer model



Danielli & Davson model



modern membrane concepts



removal of these proteins from the membrane requires detergents to disrupt hydrophobic interactions with the lipid bilayer, leading inevitably to the complete disruption of the membrane.

Peripheral proteins are removed from the membrane by mild treatments such as increasing ionic strength or addition of a chelating agent. The removal of these proteins by such mild procedures indicate weak hydrophilic associations with the polar lipid groups or polar regions of other membrane proteins.

In this scheme the carbohydrate residues are covalently bound to protein (glycoprotein) and to lipid (glycolipid) and appear to be asymmetrically distributed across the bilayer i.e. the carbohydrate is detectable only on the extracellular side of the membrane.

1.3.2. Cell Surfaces Changes Taking Place as a Result of Transformation

The recognition of differences in the cell surface architecture between normal cells and cells which have been rendered malignant as a result of viral transformation comes from several sources:-

1.3.2.1. Lectins

Lectins are proteins derived from the seeds of leguminous plants (some animal lectins have been reported: Roche & Monsigny, 1974) and have been the subject of several recent reviews (Lis & Sharon, 1973; Burger, 1973; Nicolson, 1974). These proteins possess binding sites with specificities for particular sugars and sugar groupings. It is this property which has been exploited for the isolation (Allan *et al.*, 1971) and location *in vivo* of glycoproteins (Inbar *et al.*, 1971). Interest in lectins has been aroused by the observation that wheat germ agglutinin (Aub *et al.*, 1963; Burger, 1969), Concanavalin A from the jack bean (Inbar & Sachs, 1969) and the lectin from soybean (Sela *et al.*, 1970) preferentially agglutinate malignant cells. This

is thought to be a result of changes in the cell membrane similar to those brought about by the action of proteases (Nicolson, 1971 and 1972).

Affinity chromatography using lectins covalently bound to CNBr activated Sepharose has been used (Allan et al., 1972; Hayman and Crumpton, 1972) to isolate lectin receptor sites from solubilised extracts. The isolation of binding receptors has been impeded by the insolubility of cell membranes in orthodox biochemical solvents, while more drastic solubilisation procedures destroyed the biochemical activity. However, glycoproteins associated specifically with the membrane can be solubilised by treatment with 1% ($^{w}/_{v}$) deoxycholate (Hayman and Crumpton, 1972) without loss of lectin binding activity and may be subsequently isolated on a Concanavalin A-Sepharose column.

It is these properties of lectins which allow them to be used in the investigation of cell surface architecture and in the elucidation of the changes undergone by the cell in transformation, as well as in the isolation of specific membrane constituents.

1.3.2.2. Glycopeptides from Normal and Transformed Cell Membranes

A series of experiments carried out by Warren et al. (1974) provides another source of information documenting cell surface differences between normal and transformed cells.

Warren labelled normal BHK C13 cells with $\left[^{14}C\right]$-fucose, glucosamine, galactose and mannose, while labelling transformed PYY cells with the same tritiated sugars. The cells were trypsinised to remove them from the growth surface and their surface membranes isolated. The membranes from each cell line were mixed and pronase digested to remove as much glycopeptide as possible. When the digest was subsequently applied to a Sephadex G50 column and fractions counted a significant difference was observed. Both normal and transformed

membranes exhibited a lower molecular weight fraction, peak B. Transformed cell membrane digests also showed a characteristic high molecular weight fraction, peak A, of glycopeptide in the region of molecular weight 4,600 daltons, which disappeared when the digest was treated with neuraminidase prior to application onto a G50 column.

This experiment provided the first quantitative difference in the carbohydrate components of membrane glycoproteins in normal and transformed cells.

1.3.2.3. LETS Glycoprotein

There are reports (See Review by Hynes, 1975) of variation in the amounts of specific polypeptides in transformed fibroblast cells from various species. The best characterised of these polypeptides is the LETS glycoprotein: large (L), external (E), transformation (T) sensitive (S) or otherwise called galactoprotein-a. This and other less well documented peptides which disappear or increase as a result of viral or chemical transformation have been dealt with at length in the above mentioned review. The LETS glycoprotein has a molecular weight of between 210-270,000 (depending on the method of estimation) and is an integral protein since it is not removed from the membrane by chelating agents, saline or low concentrations of the non-ionic detergent TritonX-100. The protein is extremely sensitive to proteases and is interesting by virtue of its absence or marked reduction on the surfaces of transformed fibroblast cells from several species, including mouse, rat, chicken and human.

1.3.2.4. Changes in Cell Surface Glycolipids as a Result of Transformation

The cell surface changes occurring in the glycolipid of transformed BHK cells have recently been the subject of a detailed review by Hakomori (1975). These changes, which are independent of the

carcinogenic agent, may be classified as "simplication of the glycolipid pattern due to blocked synthesis", possible as a result of interference with specific glycosyl transferase activity.

1.3.3. Intracellular Membranes and their Glycoproteins

The field of intracellular membrane glycoproteins is relatively new and little documented. In 1971 Glick et al. published a quantitative comparison of the carbohydrate content of mitochondrial, nuclear and lysosomal membranes purified from L cells and compared these to the plasma membrane levels. This study suggested that most of the sialic acid of the intact cell (i.e. ~66%) was present in the surface membrane while only small amounts appeared in other purified fractions, except lysosomes where 16% of sialic acid could be accounted for. The sialic acid content of the nuclear membrane appeared to be particularly low, less than 1% of that of the plasma membrane. Very little information, however, is generally available concerning the glycoprotein composition or the function they serve in intracellular membranes.

1.3.3.1. Mitochondria

Mitochondria are known to contain carbohydrate material (De Bernard et al., 1971; Bosmann & Martin, 1969; Yamashina et al., 1965) and the bulk of this carbohydrate is solubilised on disruption of the outer membrane and is located in the intramembranous spaces.

The difficulties involved in the preparation of clean mitochondrial membrane (Wainio, 1970) have hampered progress of the study of the glycoprotein composition and location. It is known, however, that carbohydrate residues are present on the outer mitochondrial surface. Nicolson & Singer (1971) developed a technique using ferritin conjugated Concanavalin A to stain specific saccharide residues of the erythrocyte and other membranes and thus make them visible by electron microscopy. The conjugate was found to bind specifically to the outer sur-

face of (erythrocyte) membranes. This technique has been used to detect mannose, galactose and N-acetylgalactosamine on the outer mitochondrial membranes of bovine, liver cells (Nicolson et al., 1972).

Sialic acid has also been identified on mitochondrial surfaces (Bosmann et al., 1972), although perhaps due to steric considerations, only a small proportion (~15%) of the total sialic acid present is able to be released by treatment with neuraminidase. Bosmann et al. (1972) also provided some quantitative comparison of sialic acid levels in rat liver cells. Liver mitochondria contains 2 μmol (sialic acid) gm (protein)$^{-1}$ compared to 0.62 μmol (sialic acid) gm (protein)$^{-1}$ in the outer mitochondrial membrane, compared to 33 μmol (sialic acid) gm (protein)$^{-1}$ in rat liver plasma membranes.

One mitochondrial glycoprotein which has been well studied by Sottocasa et al. (1971) is the Ca^{2+} binding glycoprotein. This ia a glycoprotein of m.w. 30-33,000 (Carafoli & Sottocasa, 1974), containing 15% by wt. carbohydrate and made up of a high proportion (35%) of acidic amino acid residues and has in addition five Ca^{2+} ions mol^{-1} of purified extract bound to it. It is thought to play a role in Ca^{2+} ion transport in and out of mitochondria since the glycoprotein binds Ca^{2+} with a dissociation constant (Kd) of the same order of magnitude as the Kd for Ca^{2+} transport (Carafoli & Azzi, 1972). This involvement in transport has also been confirmed by experiments using known Ca^{2+} transport inhibitors (Sottocasa et al., 1972) and more recently by immunological methods (Panfili et al., 1976).

1.3.3.2. Lysosomes

Lysosomal membranes are thought to be the result of a "budding off" process from either the smooth ER or the golgi apparatus. Many of the lysosomal enzymes are glycoproteins, and since carbohydrate residues are added to the peptide backbone to produce glycoproteins

at the golgi, it is not unreasonable to consider their implication in lysosomal formation. As in other intracellular membranes, carbohydrate has been detected on its surface - galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid. In the region of 50-60% of the total secondary lysosomal membrane sialic acid is released by treatment with neuraminidase, indicating a ready availability of sialic acid on the lysosomal surface to the enzyme. That fraction remaining resistant to neuraminidase action may be present within gangliosides which are also present in lysosomes (Henning and Uhlenbruck, 1973). When intact lysosomes (as opposed to lysosomal membrane fractions above) are neuraminidase treated the sialic acid released is very much less, suggesting that most of the sialic acid is located on the inner face of the lysosomal membrane, (Henning and Uhlenbruck, 1973). The high sialic acid content of lysosomal membrane may be contributed by the complex carbohydrates of the plasma membrane during formation of the endocytotic lysosome.

1.3.3.3. Endoplasmic Reticulum

The ER is a membrane plexus within the cell responsible, among other things, for the synthesis and transport of glycoproteins. Much of the data available for ER comes from work done on liver cells. That part of the ER surface where protein synthesis is carried out is covered with ribosomes and is termed the rough ER; the remainder being known as the smooth ER. Quantitative differences in the carbohydrate content of the glycoproteins between the rough and smooth ER have been reported (Dallner and Azzi, 1972). The rough ER is thought to contain a higher level of mannose than the smooth, while the latter may contain more galactose and sialic acid.

1.3.3.4. The Nuclear Envelope

The nuclear envelope, the double membrane separating the

chromatin from the cytoplasm was identified microscopically in the 1950's. To what extent the nuclear membrane is involved in the control of metabolite mobility passing in and out of the nucleus remains an unexplored field of study. Our understanding of the function of the nuclear envelope, an elucidation of its chemical composition and activity will necessarily be dependent on its isolation in a pure form. A complete review of the methods of isolation are available (Birnie, 1976; Maddy, 1976).

There being no enzyme markers peculiar to the nuclear membrane, it remains unclear whether parts of the inner nuclear envelope are attached to chromosomal material on the one side, or are continuous with the cellular endoplasmic reticulum on the other.

Permeability studies on intact nuclei (Fry, 1970; Feldherr, 1970) have shown that small ions and some macromolecules can move freely across the nuclear envelope. It is not known, however, by what mechanism RNA or ribonuclear protein move into the cytoplasm, nor what feedback control there is from the cytoplasm to control gene function in the nucleus.

It is claimed that the inner and outer nuclear envelope may be structurally dissimilar, the latter bearing ribosomes and being removable by non-ionic surfactants such as TritonX-100 (Sadowski and Howden, 1968) or citric acid (Smith et al., 1969), while the inner nuclear envelope remains intact. Recently, however, Aaronson & Blobel (1974) found that Triton X100 completely removed the double membrane envelope from isolated nuclei and that such membrane denuded nuclei retained both their shape and ultrastructure. They also demonstrated that the pore complex was not removed as a result and may be responsible for the maintainance of the nuclear structure.

Further, it has been shown (Stuart et al., 1977) that the trans-

fer of nucleic acids from isolated nuclei to the surrounding medium is independent of the nuclear membrane.

To date little work has been carried out on the chemical characterisation of nuclear glycoproteins. Kashnig and Kasper (1969) working on rat liver cells separated nuclear membrane proteins into their various molecular weight classes by SDS polyacrylamide gel electrophoresis. Using different staining techniques they located a major glycoprotein band in the molecular weight region of 150,000 (4.2% of total nuclear envelope protein), and two or three minor proteins in the molecular weight range of 50-74,000.

Some characterisation of chick erythrocyte nuclear membrane has been carried out by Jackson (1976) using similar techniques. He describes four major polypeptides of molecular weights 80,000, 74,000, 62,000 and 50,000. Lesser bands of molecular weight 29,000, 14,000 and 10,000 are ascribed to histone contamination. He concludes

i) that the nuclear envelope SDS gel pattern differed radically from the erythrocyte plasma membrane pattern

ii) on the basis of salt extraction (0.1M NaCl) the 80 and 74K* polypeptides are peripheral (extracted) proteins, while the 62 and 50 K are integral (remain membrane bound)

iii) the 80, 74 and 62K bands are common to bands isolated from liver cells

iv) on the basis of Schiff staining, the erythrocyte nuclear membrane contains no glycopeptides.

A recent paper by Seiber-Blum & Burger (1977) suggested that the nuclear envelope of CHO cells can be resolved into 47 polypeptide bands by electrophoresis on SDS polyacrylamide gels. Nine of these

*K = 10^3

bands appeared to contain amino sugars. Examination of their protein at different stages of the cell cycle revealed that only five of the 47 bands showed any difference in incorporation of radioactively labelled leucine, glucosamine, indicating that the nuclear envelope proteins are synthesised de novo during mitosis, but that the envelope is probably assembled from existing membrane.

No work has yet been published which gives a precise account of nuclear glycoproteins or compares the glycoprotein changes which may take place in the nuclear envelope after transformation by oncogenic viruses.

To this end this thesis describes the methods developed for the preparation of the nuclear envelope and contains the findings observed on the basis of SDS polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

2.1. Materials

The following materials were obtained from the Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri, U.S.A.

Cytosine arabinoside (1-β-D-arabinofuranosyl cytosine)

2-Deoxyglucose (grade II)

DNAse II (Deoxyribonuclease II EC 3.1.4.6) ex bovine spleen
Lot No. 53C 1940

DOC (sodium salt of 7-deoxycholic acid)

Dowex-1 Ion Exchange Resin 1 x 8-100, 50-100 mesh
(chloride form)

Hyaluronidase (EC 3.2.1.35) Type VI ex bovine testis

α-Methylglucoside (1-O-methyl-α-D-glucopyranoside)

MOPS (morpholinopropane sulphonic acid)

DL-Norleucine (Sigma grade)

PMSF (phenylmethylsulphonyl fluoride)

Pyruvic acid

Triton X-100(p-t-octylphenoxypolyethoxyethanol)

Trizma base, Tris (Hydroxymethyl) amino methane (Reagent grade)

In addition to the following, all other laboratory reagents not specifically mentioned were of 'Analar' grade and bought from the British Drug Houses Chemicals Ltd., Poole, England:-

Dimethyl sulphoxide (DMSO)

Hydroxyurea

2-Mercaptoethanol

N N'—Methylenebisacrylamide (specially purified for electrophoresis)

N N N'N'—Tetramethylenediamine (TEMED)

Nonidet P40

Pronase (EC 3.4.24.4)

Sodium dodecyl sulphate

Toluene, 'Analar'; methane sulphonic acid (MSA) and Tween 80 were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

Flow Laboratories, Paisley, Scotland were the suppliers of

MEM (minimal essential medium) Amino acids X50

MEM Vitamins X100

Foetal calf serum and calf serum

Streptomycin

Penicillin

The following chemicals are listed in alphabetical order of the supplier:-

Crystalline bovine serum albumin (protein standard solution)

Armour Pharmaceuticals Co., Chicago, Illinois, U.S.A.

DNAase I (Deoxyribonuclease I, EC 3.1.4.5) ex bovine pancrease (B grade) Lot No. 000148

Cal. Biochem. Ltd., Thorpe House, King Street, Hereford, England.

Bis MSB Bis (O-methyl-steryl) benzene (scintillation grade)

Eastman Kodak Co., Rochester, N. York, U.S.A.

Heparin (freeze, dried, pyrogen-free)

Evans Medical Ltd., Speke, Liverpool, England.

Pyronin Y

George T. Gurr, London, England.

Coomassie Brilliant Blue R

Gurr/Searle Diagnostic, High Wycombe, Bucks., England.

n-Butyl acetate

Hopkins and Williams, Chadwell Heath, Essex, England.

PPO (2,5-diphenyloxazole) (scintillation grade)

International Enzymes, Ltd., Windsor, Berkshire, England.

Protosol (Tissue and Gel Solubiliser)

New England Nuclear, Boston, Mass., U.S.A.

Hyamine hydroxide

Nuclear Chicago, G.D. Searle, High Wycombe, Bucks., England.

Sephadex G50 (fine) and Sepharose 4B

Pharmacia Fine Chemicals Ltd., Uppsala, Sweden.

Triton X100 (bought in bulk for liquid scintillation counting)

Rohm and Haas (UK) Ltd., 2 Masons Avenue, Croydon, England.

DEAE-cellulose (diethylamino ethyl cellulose) (DE 32)

microgranular form

Whatman, W. & R. Balston, Springfield Mill, Maidstone, Kent, England.

The following radioactive isotopes were purchased from the Radiochemical Centre, Amersham, England.

D-$[6\text{-}^{3}H]$ Glucosamine HCl (aqueous solution)

specific activity (sp. act.) 20 Ci $mmol^{-1}$

L-$[1\text{-}^{3}H]$ Fucose (aqueous solution)

sp. act. 33 Ci $mmol^{-1}$

$[1\text{-}^{3}H]$ Ethan-1-ol-2-amine (aqueous solution)

sp. act. 3.8 Ci $mmol^{-1}$

L-$[4,5\text{-}^{3}H]$ Leucine

sp. act. 60 Ci $mmol^{-1}$

$[U\text{-}^{14}C]$ Protein hydrolysate (aqueous solution containing 2% ethanol)

sp. act. 54 mCi $m.Atom^{-1}$

2.2. Methods

2.2.1. Methods in Tissue Culture

Solutions in tissue culture experiments were made up in the Cell Culture Unit at Glasgow University as follows:-

Earle's Balance Salt Solution (BSS)

	gl^{-1}
NaCl	68
KCl	4
$MgSO_4.7H_2O$	2
$NaH_2PO_4.2H_2O$	1.4
$CaCl_2.6H_2O$	3.93
Phenol red, 1%	15 ml
Distilled H_2O	1 litre

Before use, 50 ml of the above is diluted to 450 ml with distilled H_2O and autoclaved at 15 lb in^{-2} pressure for 20 min.

Eagle's stock solution 10 x concentrated (ES x 10)

		per litre
MEM	amino acids x50	200 ml
MEM	vitamins x100	200 ml
Glucose		45 g
Distilled H_2O		1 litre

The pH was adjusted to 7.1 using 5N NaOH before sterilisation by Millipore filtration using a G5 membrane (0.22 μ).

Eagle's stock was monitored for bacterial contamination in

a) Saboraud fluid medium at 31°C for 1 week

b) Brain heart infusion broth at 37°C for 1 week

and subsequently stored at 4°C.

Penicillin and Streptomycin (P/S)

	per litre
Penicillin	10^7, units
Streptomycin	10 g
Distilled H_2O	1 litre

Sterilised and checked as for Eagle's stock and stored in 5 ml Bijoux bottles at -20°C.

Sodium bicarbonate solution

5.6% sodium bicarbonate with 1% phenol red was sterilised and checked for bacterial contamination as above and stored at room temperature in tightly closed metal-capped universal bottles.

Serum

Calf serum and foetal calf serum were stored at -20°C until used.

Eagle's medium

Eagle's medium supplemented with 10% calf serum (EC 10) or foetal calf serum (EFC 10) was freshly prepared for each cell growth experiment as follows:-

BSS	450 ml
ES x10	50 ml
Na HCO_3	20 ml
Serum	50 ml
P/S	5 ml

Glucose-free EC 10/EFC 10 was prepared by making up glucose-free ES x10 and supplementing the resulting EC 10/EFC 10 with 50 mM pyruvic acid.

Harvesting solution

BSS	450 ml
Na HCO_3/phenol red	20 ml

Seeding, Growth and Labelling of BHK Cells

Unless otherwise stated BHK C13 and PYY cells were seeded at a concentration of 30 x 10^6 cells in roller bottles containing 180 ml normal EC 10 and maintained, slowly rotating, in a horizontal position at 37°C. When the cells had reached a logarithmic growth phase and before reaching confluent growth (after approximately 24 h), they were labelled aseptically with sterile radioactive isotopes as specified for each experiment. The cells were harvested by washing three times with cold (0°C) harvesting solution and were scraped from the sides of the roller bottle into a volume (approximately 20 ml) of the same solution.

2.2.2. Separation Techniques

2.2.2.1. Gel Filtration

Sephadex G50 (fine) (24.0 x 1.5 cm) was used to separate the high and low molecular weight species of extracellular secreted material. The elution buffer was 10 mM Tris pH 8.2 containing 0.15M KCl, the flow rate was 11.5 ml h^{-1} cm^{-2}.

2.2.2.2. Ion Exchange Chromatography

Extracellular mucopolysaccharides were separated on diethyl-aminoethyl cellulose as described by Kraemer (1971). The DEAE-cellulose (DE 32) was pre-treated with acid and alkali in accordance with the maker's instructions. A column (Pharmacia) (10.0 x 0.8 cm) was packed and equilibrated with 0.01M ammonium acetate and eluted with a linear gradient from 0.01M to 2M ammonium acetate. Fractions (4 ml) were collected and 1 ml aliquots from each taken for radioactive counting.

2.2.2.3. Sodium Dodecyl Sulphate Gel Electrophoresis

Gel electrophoresis of intact nuclei and nuclear membranes was carried out as described by Fairbanks et al. (1971)

Nuclear membrane samples containing approximately 0.1 mg protein were incubated in buffer containing 1% ($^{v}/_{v}$) mercaptoethanol reducing

agent and 1% ($^{w}/_{v}$) SDS at 100°C for 10 min before application to polyacrylamide gels (10.0 x 0.6 cm).

Coomassie blue stained gels were scanned at 550 nm using a Gilford spectrophotometer model 240 with a linear transport attachment model 2410 (Gilford Instruments Ltd., Teddington, Middlesex, England).

Stained or unstained radioactive gels were sliced into 1 mm slices for radioactive counting, using a Mickle gel slicer (Mickle Laboratory Engineering Co., Gernshall, Surrey, England).

2.2.3. Liquid Scintillation Counting

2.2.3.1. Aqueous Samples

Aqueous radioactive samples were prepared for counting by addition of Triton-toluene (9 ml) to 1 ml of sample. Triton-toluene scintillation fluid for such samples had the following composition:- 37.5% ($^{v}/_{v}$) TritonX-100 65% ($^{v}/_{v}$) toluene, 0.5% ($^{w}/_{v}$) PPO and 0.05% ($^{w}/_{v}$) Bis MSB.

2.2.3.2. Non-aqueous Samples

Non-aqueous samples were evaporated to dryness in a fume cupboard. Hyamine hydroxide (1 ml) was added and the samples incubated for 1 h at 60°C before counting in toluene-PPO (9 ml). Toluene-PPO scintillation fluid contained 0.5% ($^{w}/_{v}$) PPO in toluene.

2.2.3.3. SDS-Polyacrylamide Gels

Gel slices (1 mm each) were incubated at 37°C overnight in Protosol (4.5 ml) diluted to a 3% ($^{v}/_{v}$) solution with toluene-PPO and counted in plastic disposable 'mini-vials'.

2.2.4. Preparation of Nuclei

2.2.4.1. Detergent Method

Growing BHK cells were harvested as described in section 2.2.1. The cells were energetically pipetted in the harvesting solution until a homogeneous suspension of cells resulted. These were pelleted by

centrifugation at 1000xg (av.) 5 min at 4°C and the pellet resuspended in 0.5 ml solution containing 0.5 ($^{v}/_{v}$) Nonidet P40 , 0.25M sucrose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 25 mM Tris pH 8.0. The suspension was centrifuged at 1000xg (av.) for 10 min at 4°C through a cushion of 0.32M sucrose.

2.2.4.2. Non-Detergent Method

Growing cells (Cl3 and PYY) were harvested as described in section 2.2.1. and centrifuged for 5 min at 600xg (av.). The pellet was resuspended in sucrose/tris solution (1M sucrose (100 ml), 0.8M Tris pH 7.5 (10 ml), 1M $CaCl_2$ (1.2 ml) and distilled H_2O (289 ml)) and the suspension was recentrifuged. This was repeated. The pellet was then resuspended in a volume of sucrose/tris ten times that of the pellet; homogenised (5 passes in a teflon in glass Potter homogeniser, the teflon piston was rotated by an electric motor) and the cells checked microscopically for breakage. If breakage was 90% complete the suspension was centrifuged at 1000xg (av.) for 10 min at 4°C. The supernatant fluid was retained for the preparation of endoplasmic reticulum. The pellet was washed four times with sucrose/tris solution and centrifuged each time for 10 min at 1000xg (av.) 4°C. The final pellet was observed using phase contrast microscopy.

2.2.5. Preparation of Nuclear Membrane

2.2.5.1. Heparin Method

In this preparation PMSF was made up 200 mM in DMSO and was added to all solutions at a final concentration of 0.1 mM.

Nuclei were prepared by the non-detergent method as described in section 2.2.4.2. and lysed by freezing and thawing three times in 0.02M Tris pH 7.2 (1 ml). Sucrose was subsequently added to give a final sucrose concentration of 0.25M and the volume made up to 1.5 ml. To this was added 0.02M Na_2HPO_4 (0.17 ml) and heparin (400 µl of a

10 mg ml^{-1} solution). The mixture was agitated using a vortex mixer and subsequently kept on ice for 10 min. Pancreatic DNAse I (1 mg) and bovine spleen DNAse II (1 mg) were added to the mixture. This was followed by an incubation period of 30 min on ice before addition of H_2O (5 ml). The thoroughly mixed solution was centrifuged through a 54% ($^{w}/_{w}$) sucrose cushion in an SW40 rotor at 200,000xg (av.) for 2 h (4°C) in a Beckman L565 centrifuge.

The resulting material which banded at the interphase of the two solutions was pelletted in a Beckman Ti50 rotor centrifuged at 160,000xg (av.) for 45 min (4°C). This crude nuclear membrane pellet was resuspended in 0.02M Tris pH 7.2 (1 ml) and layered onto a sucrose density gradient made up of the following concentration steps 50%, 43% and 37% ($^{w}/_{w}$) and centrifuged overnight at 200,000xg (av.) in a Beckman SW40 rotor at 4°C.

2.2.5.2. Preparation of Nuclear Membrane by Sonication

The nuclear pellet obtained as a result of the non-detergent nuclear preparation (2.2.4.2.) was resuspended in TKM buffer pH 7.5 (5 ml) (0.05M Tris pH 7.5, 0.025M KCl and 0.005M $MgCl_2$). The nuclei were disrupted by sonication in a bath for 5 min at maximum power. When 80% of the nuclei were seen to be lysed (phase contrast microscopy), DNAse I (1 mg) and DNAse II (1 mg) were added and the mixture incubated at 0°C for 30 min. The sample was thoroughly mixed, then centrifuged through a 54% ($^{w}/_{w}$) sucrose barrier as above. The material which banded at the interphase of the two solutions was removed, pelletted by centrifugation, resuspended in TKM (1 ml) and applied to a step-wise sucrose gradient as was carried out for the method described in section 2.2.5.1.

2.2.6. Preparation of Endoplasmic Reticulum

The supernatant fluid resulting from the crude nuclear preparation (section 2.2.4.2.: supernatant from centrifugation prior to the final 4 washings) was removed and centrifuged for 10 min at 10,000 g (av.) in

an 8 x 50 ml angle rotor in the MSE High Speed 18 centrifuge. The resulting supernatant is then centrifuged at 160,000 g (av.) for 1 h (4°C) in a Beckman Ti 50 angle head in order to pellet endoplasmic reticulum.

2.2.7. Determination of Protein

Estimation of nuclear membrane protein was carried out using the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.2.8. Determination of Amino Sugars

The release of N-acetylgalactosamine and N-acetylglucosamine from nuclear membrane bands was carried out in 4M methane sulphonic acid at 100°C for 70 h in evacuated, sealed and previously acid-washed Pyrex tubes.

The membrane sample, suspended in water (0.5 ml) was made up to a volume of 1 ml with 8M methane sulphonic acid (final concentration 4M in methane sulphonic acid). DL-Norleucine (50 nmol) was added as an internal standard. After hydrolysis, 3.5M sodium hydroxide (0.5 ml) and 1M sodium hydroxide (0.2 ml) were added. This was followed by further small additions of alkali until the pH became 2-2.5.

The resulting solution was analysed in an amino acid analyser JLC-JAH (Japanese Electron Optics Laboratory Co. Ltd., Tokyo, Japan) using 10 cm and 50 cm columns of LCR1 resin.

2.2.9. Chromatography of Sialic Acid

Sialic acid was released by hydrolysis of [^{3}H]-glucosamine labelled membrane in 0.05M H_2SO_4 at 80°C for 1 h and separated on Dowex 1-8X (formate form) by elution with 0.3M formic acid. Further separation was carried out by descending paper chromatograph (Whatman No. 1 paper) in n-butylacetate - acetic acid - water (3:2:1 by volume). N-acetylneuraminic acid was detected using silver nitrate as follows:-

i) the paper was dipped in a solution of silver nitrate in acetone (50% saturated $AgNO_3$ (0.1 ml) in acetone (25 ml))

ii) the paper was dried and rinsed in alkaline ethanol (0.5M NaOH in 45% ($^{v}/_{v}$) ethanol)

iii) after the development of the spots the dark background was removed by washing in 1% ($^{w}/_{v}$) sodium thiosulphate and a final washing in water before drying.

A chromatographic standard of N-acetylneuraminic acid was used simultaneously with the membrane sample. The staining procedure was carried out on the standard only. That part of the chromatogram containing the radioactive sample was sliced into 1 cm strips and counted in a liquid scintillation counter using hyamine hydroxide and toluene PPO scintillation fluid as described in section 2.2.3.2.

2.2.10. Lectin Binding Studies

The haemagglutinin from lentils (Lens culinaris) was prepared in the laboratory from a method based on that of Agrawal and Goldstein (1967).

The cyanogen bromide coupling of the lentil lectin to Sepharose 4B was carried out as described in the instructions provided by Pharmacia.

Binding studies were carried out as follows. The sample under investigation was dissolved in 1% ($^{w}/_{v}$) DOC (1 ml). A Pharmacia glass column (8.0 x 0.8 cm) was packed with the prepared sepharose-bound lentil lectin and washed initially in buffer containing 20 mM MOPS pH 8.2, 0.15M KCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.4% ($^{w}/_{v}$) NaN_3.

Since the DOC in the eluting buffer caused precipitation of Mn^{2+} and Ca^{2+} ions required for binding, the column was washed with buffer containing 20 mM MOPS, 0.15M KCl and 0.4% ($^{w}/_{v}$) NaN_3 prior to brief washing with the eluting buffer of 20 mM MOPS, 0.15M KCl and 1% ($^{w}/_{v}$) DOC.

The sample was loaded onto the column (flow rate 19.8 ml h^{-1} cm^{-2})

after the passage of three column volumes the material which had bound to the column was eluted with eluting buffer (20 mM MOPS, 0.15M KCl 1% ($^{w}/_{v}$) DOC containing 0.25M α-methylglucoside as a competing sugar).

2.2.11. Electron Microscopy

Nuclear membrane from BHK C13 and PYY cells was prepared as previously described (section 2.2.5.1.). The three membrane bands from each preparation isolated on a sucrose density gradient were washed in 0.02M Tris buffer pH 7.2 and pelletted by centrifugation in a Beckman Ti 50 rotor at 100,000xg (av.) for 30 min.

The pellets were fixed at pH 7.2 following the method of Sabatini et al. (1964) i.e. subjected to 2% glutaraldehyde in 0.02M Tris pH 7.2 for 1 h, followed by 2 h in 0.02M Tris and 1 h in 1% osmium tetroxide in 0.02M Tris pH 7.2.

The samples were dehydrated in graded acetone; cleared in propylene oxide and embedded in Epon (Luft, 1961). Sections (50 nm) were cut on an L.K.B. ultrotome III using a Dehmar diamond knife, and mounted on Fomvar coated copper grids. The sections were stained with saturated alcoholic uranyl acetate and Reynold's lead citrate (Reynolds, 1963). Examination of the stained sections was carried out on an A.E.I., E.M.6B electron microscope at an accelerating voltage of 60 Kv. Selected areas were photographed on Ilford SP332 film which was developed in May and Baker Qualitol developer.

RESULTS

3.1. Inhibitor Studies

3.1.1. The Effect of Adding Unlabelled Glucosamine on the Incorporation of $[^3H]$-Glucosamine into Intracellular Pools and Intracellular and Extracellular Glycoproteins

Petri dishes were seeded and treated as described in the legend for Fig. 3. Three fractions were investigated as follows:-

The growth medium was removed at the end of the 4 h labelling period and precipitated in the cold (0^oC) with trichloroacetic acid (TCA final concentration 5% ($^w/_v$)). The resulting suspension was centrifuged at 9,000xg (av.) for 10 min (MSE High Speed 18 using an 8 x 50 rotor). The precipitate was then washed five times and the final precipitate dissolved in 0.1M NaOH (1 ml), and neutralised with 0.1M HCl (1 ml). Aliquots of this (1 ml) were counted in a Phillips Liquid Scintillation Analyser using Triton X-100 scintillation fluid.

Immediately after the removal of the growth medium the cells were washed three times in aliquots (3 ml) of ice-cold Earle's Balanced Salt Solution (containing sodium bicarbonate) in order to remove excess growth medium. The cells were then washed three times with cold 5% ($^w/_v$) TCA (3 ml). The radioactivity in the first TCA wash was determined by scintillation counting of 1 ml fractions. This determination gave an indication of $[^3H]$-glucosamine incorporated into the intracellular TCA-soluble glucosamine pool.

The remaining acid-precipitated material was solubilised by incubation in 0.1M NaOH (1 ml) at 37^oC for 1 h, followed by neutralisation and radioactive counting as described above.

The results obtained are shown in Fig. 3. Addition of unlabelled glucosamine in the concentrations shown appears to have had no effect on the incorporation of $[^3H]$-glucosamine into the intracellular pools of glucosamine or intracellular glycoproteins. This indicates a dilution

FIGURE 3 The Effect of Adding Unlabelled Glucosamine on the Incorporation of $[^3H]$-Glucosamine into Intracellular Pools and Intracellular and Extracellular Glycoproteins

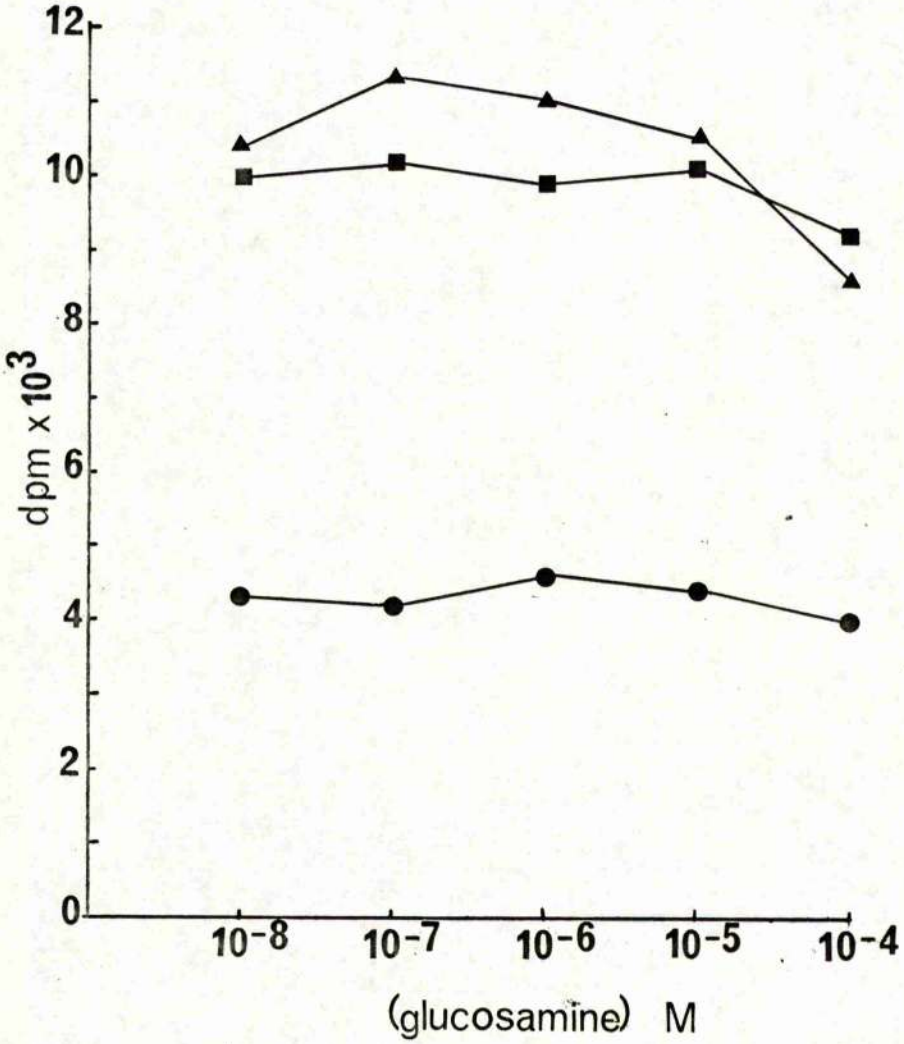
5×10^5 BHK C13 cells were seeded in EFC 10 medium (3 ml), glucose-free and 50 mM in pyruvate. After 18 h, the cells were labelled with $[^3H]$-glucosamine (2 μCi) in varying concentrations of unlabelled glucosamine for a period of 4 h. The following were monitored for radioactivity at the end of this 4 h incubation:-

1) the growth medium (TCA precipitable material) ●—●—●

2) the TCA cell washing, giving the TCA soluble fraction ■—■—■

3) the TCA precipitable fraction ▲—▲—▲

Each experimental point shown is the mean of duplicate determinations.


12
10
8
6
4
2
0
dpm x 10^3
10^-8
10^-7
10^-6
10^-5
10^-4
(glucosamine) M

of the $[^3H]$-glucosamine by the intracellular glucosamine pool.

3.1.2. The Effect of Hydroxyurea on the Time Course of Incorporation of $[^3H]$-Glucosamine and $[^3H]$-Leucine into Intracellular Glycoproteins

The experiment was carried out as described in the legend for Fig. 4. This Figure illustrates that at a concentration of 10^{-2}M, hydroxyurea had an insignificant effect on the incorporation of $[^3H]$-leucine. On the other hand, a limited inhibition of $[^3H]$-glucosamine incorporation (33-40%) into TCA precipitable material was observed.

It may be noted that although the ratio of $[^3H]$-leucine to $[^3H]$-glucosamine added to the cells was 1:10, the ratio of radioactive counts actually incorporated into the TCA precipitable cellular material was reversed.

3.1.3. The Effect of Hydroxyurea after a 16 h Period on the Incorporation of $[^3H]$-Glucosamine and $[^3H]$-Leucine into Intracellular Material

This experiment was carried out as described in the legend for Table 1. This table immediately demonstrates the long term inhibitory effects of hydroxyurea on both protein and glycoprotein biosynthesis, those being 91% and 87% respectively. It should be noticed that the long term effects of hydroxyurea are non-selective in contrast to the case observed in the short term experiment depicted in Fig. 4 (viz. $[^3H]$-glucosamine incorporation only was inhibited).

3.1.4. The Effect of Cytosine Arabinoside on $[^3H]$-Glucosamine Incorporation into BHK Cells

It is evident from Fig. 5 that at a concentration of 30 mM, over a period of 5 h cytosine arabinoside did not appear to inhibit the incorporation of $[^3H]$-glucosamine into BHK cells. The slight decrease (11%) of incorporation in the presence of inhibitor after 7 hours was

FIGURE 4 The Effect of Hydroxyurea (10^{-2}M) on the Time Course of Incorporation of [^{3}H]-Glucosamine and [^{3}H]-Leucine into Intracellular Glycoproteins

Petri dishes (50 mm) containing normal EFC 10 medium (3 ml) were set up in duplicate pairs and seeded with BHK cells (5×10^5). After 18 h of cell growth, inhibitor (previously sterilised by filtration) was introduced into the external growth medium of one of each pair. Radioactive label (20 μCi [^{3}H]-glucosamine or 2 μCi [^{3}H]-leucine) was introduced to a pair of dishes (with and without inhibitor) for a period of 1 h.

At the end of the 1 h labelling period, the radioactivity in the TCA precipitable fraction was counted as described in section 3.1.1.

Incorporation of [^{3}H]-leucine, no inhibitor ●—●—●

Incorporation of [^{3}H]-leucine, with inhibitor ⊙—⊙—⊙

Incorporation of [^{3}H]-glucosamine, no inhibitor ▲—▲—▲

Incorporation of [^{3}H]-glucosamine, with inhibitor △—△—△

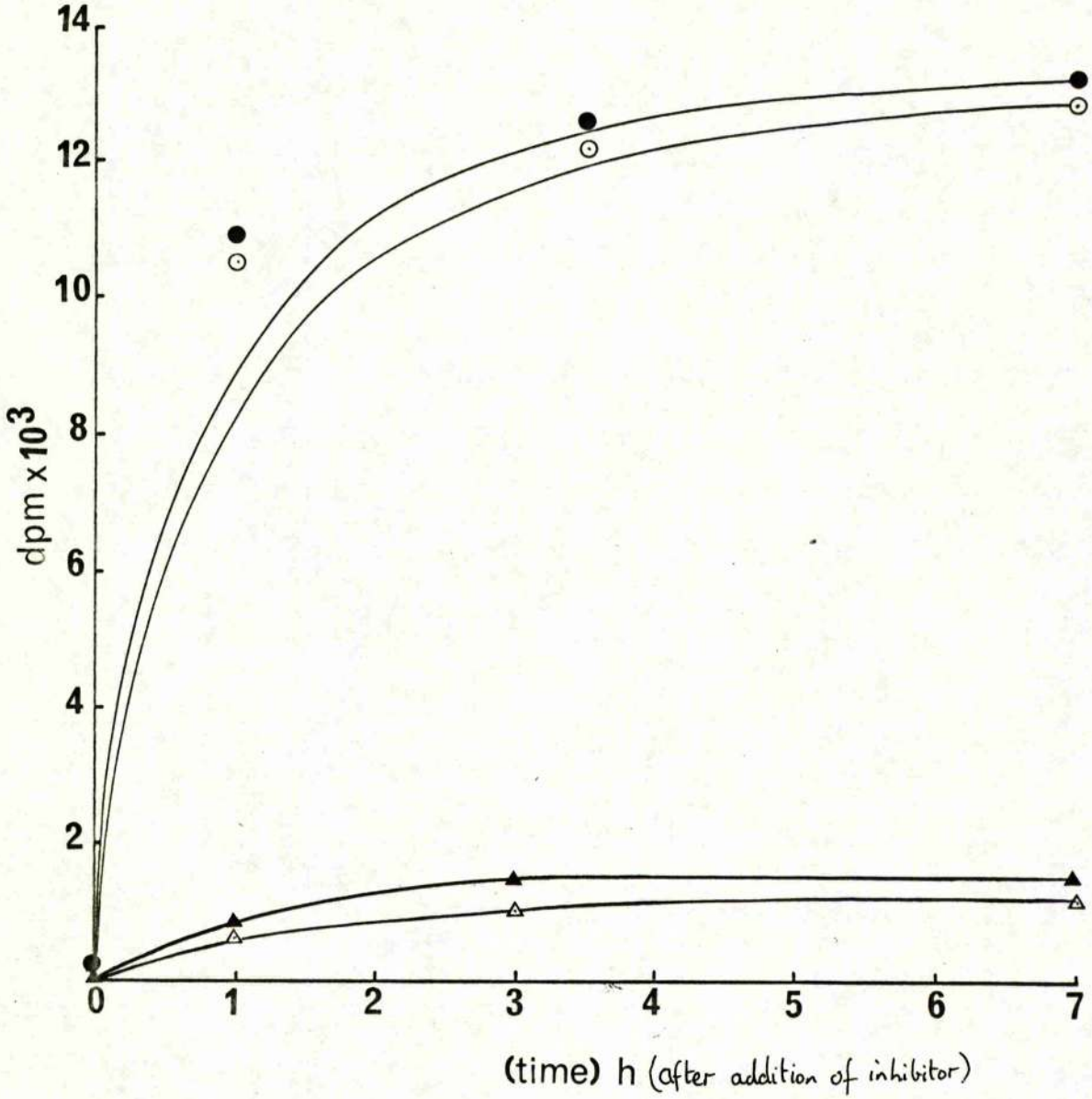

14
12
10
8
6
4
2
dpm x10^3
0
1
2
3
4
5
6
7
(time) h (after addition of inhibitor)

TABLE 1

The Effect of Hydroxyurea on the Incorporation of $[^3H]$-Glucosamine and $[^3H]$-Leucine into Intracellular Material

Inhibitor	$[^3H]$-glucosamine incorporated (dpm)	$[^3H]$-leucine incorporated (dpm)
-	41,028	121,013
+	5,483	10,614
% Inhibition	87	91

BHK cells were seeded as described in section 3.1.1. Hydroxyurea (final concentration 10^{-2}M) was added 16 h prior to a 1 h incubation period with $[^3H]$-glucosamine (200 μCi) or $[^3H]$-leucine (20 μCi). TCA precipitable radioactivity was monitored as for other experiments in this section.

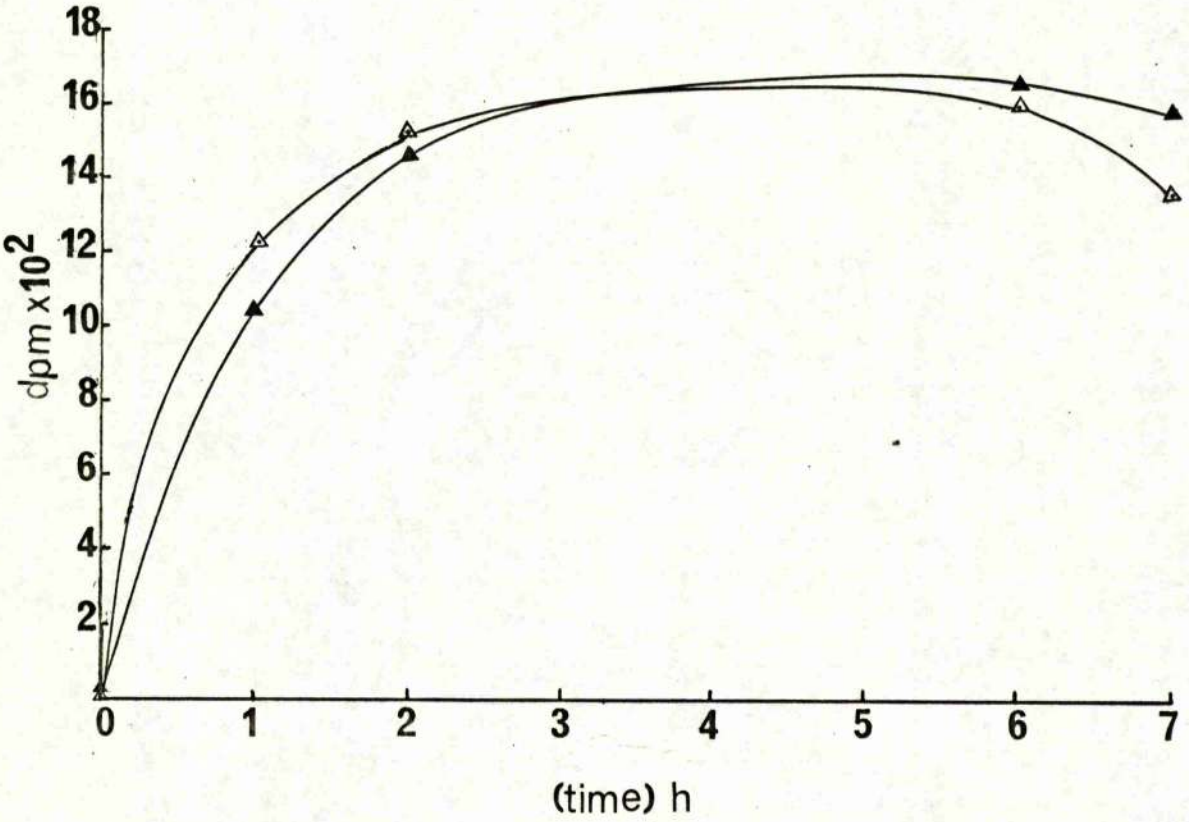
FIGURE 5 The Effect of Cytosine Arabinoside on $[^3H]$-Glucosamine Incorporation into BHK Cells

This experiment was carried out as in 3.1.1. for the investigation of the incorporation of $[^3H]$-glucosamine into acid precipitable material intracellularly. The final concentration of cytosine arabinoside in the external medium was 3×10^{-2}M.

Fig. 5 shows mean values of duplicate results.

Incorporation of $[^3H]$-glucosamine, with inhibitor △—△—△

Incorporation of $[^3H]$-glucosamine, without inhibitor ▲—▲—▲


18
16
14
12
10
8
6
4
2
dpm x10²
0
1
2
3
4
5
6
7
(time) h

considered to be within the limits of experimental error.

3.1.5. The Effect of 2-Deoxyglucose Concentration on [^{3}H]-Glucosamine and [^{3}H]-Leucine Incorporation into Intracellular Acid Precipitable Material

Fig. 6 shows that the presence of 2-deoxyglucose in concentrations 10^{-3}M had small effect (15% inhibition) on the intracellular incorporation of both [^{3}H]-glucosamine and [^{3}H]-leucine. As the concentration increased, however, so did the inhibition of radioactive incorporation, reaching a maximum of 54% at 3×10^{-2}M in the case of [^{3}H]-leucine; and 82% at the same concentration in the case of [^{3}H]-glucosamine incorporation.

The concentration of 2-deoxyglucose chosen for subsequent experiments (2×10^{-2}M) was within the range of high inhibitory effect.

3.1.6. The Effect of 2-Deoxyglucose on the Time Course of Incorporation of [^{3}H]-Glucosamine and [^{3}H]-Leucine into Intracellular Glycoproteins

Table 2 (a and b) confirms the result obtained in Fig. 6 that at the end of 1 h incubation in the presence of deoxyglucose (final concentration 0.02M in the growth medium), the inhibition of [^{3}H]-glucosamine incorporation was high (86%) and relatively lower (43%) in the case of [^{3}H]-leucine incorporation, although the amount of [^{3}H]-leucine added to the cells was 10x greater than that of [^{3}H]-glucosamine.

It was also found from this experiment that the percentage inhibition in each case increased as the length of time of exposure to the inhibitor was increased, reaching a maximum at the end of 6 h i.e. 96% inhibition of [^{3}H]-glucosamine incorporation and 64% inhibition of [^{3}H]-leucine incorporation.

FIGURE 6 The Effect of 2-Deoxyglucose Concentration on $[^3H]$-Glucosamine and $[^3H]$-Leucine Incorporation into Intracellular Acid Precipitable Material

Petri dishes (50 mM) containing EFC 10 medium (3 ml), glucose-free and 50 mM in pyruvate, were set up in duplicate pairs and seeded with BHK cells at a concentration of 5×10^5 cells. After 18 h of cell growth different concentrations of 2-deoxyglucose were added to each pair 6 h prior to introduction of $[^3H]$-glucosamine (20 μCi) or $[^3H]$-leucine (2 μCi).

At the end of the 1 h labelling period the effect of the deoxyglucose was determined as radioactive disintegrations in the intracellular TCA-precipitable fraction.

Incorporation of $[^3H]$-glucosamine ▲——▲——▲

Incorporation of $[^3H]$-leucine ●——●——●

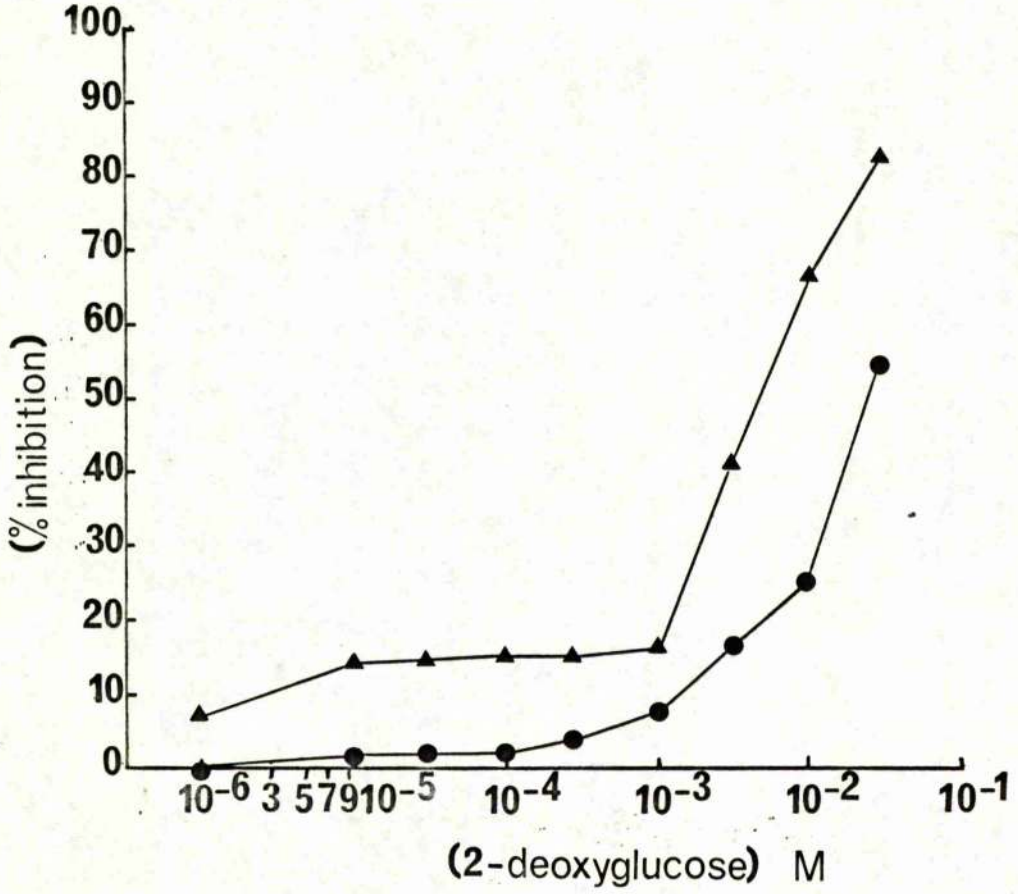

100
90
80
70
60
50
40
30
20
10
0
(% inhibition)
10-6 3 5 7 9 10-5
10-4
10-3
10-2
10-1
(2-deoxyglucose) M

TABLE 2

The Effect of 2-Deoxyglucose on the Time Course of Incorporation of $[^3H]$-Glucosamine and $[^3H]$-Leucine into Intracellular Glycoproteins

a) Incorporation of $[^3H]$-Glucosamine

Labelling period (h)	1-2	2-3	5-6	6-7
-I (dpm)	4,900	6,300	9,100	11,100
+I "	700	450	400	1,100
% Inhibition	86	93	96	90

b) Incorporation of $[^3H]$-Leucine

Labelling period (h)	1-2	2-3	5-6	6-7
-I (dpm)	11,350	10,025	16,949	14,875
+I "	6,450	5,301	6,101	5,900
% Inhibition	43	47	64	60

The concentration of 2-deoxyglucose in each case was 0.02M. The experiment was carried out as described in the legend for Fig. 4, except that the growth medium used in this experiment was EFC 10, glucose free and 50 mM in pyruvate.

3.1.7. The Effect of 2-Deoxyglucose on the Time Course of Incorporation of $[^3H]$-Glucosamine into BHK Cells Grown on Normal EFC 10 Medium

It is seen from Table 3 that the effect of 2-deoxyglucose in normal medium substantially reduced $[^3H]$-glucosamine incorporation by an average of 27% as compared with Table 2(a).

The experiment was carried out on normal EFC 10 (containing glucose) to confirm that inhibition of $[^3H]$-glucosamine uptake was more efficient in glucose-free EFC 10. Deoxyglucose is phosphorylated intracellularly and thus causes depletion of the intracellular nucleotide pools necessary to glucose phosphorylation and intermediary metabolism. The addition of pyruvate as a substrate in glucose-free EFC 10 allows the build up of phosphorylated deoxyglucose, whilst still providing the cell with a carbon source.

3.1.8. The Effect of 2-Deoxyglucose on the Intracellular Incorporation and Extracellular Secretion of $[^3H]$-Glucosamine

Table 4 shows the marked inhibition (71%) of $[^3H]$-glucosamine incorporation into intracellular TCA-precipitable material; and a slightly less (60%) inhibition of incorporation into the TCA soluble pool.

It would appear that 2-deoxyglucose had an insignificant effect (8% inhibition) on the extracellular secretion of $[^3H]$-glucosamine or glycosylated products, as monitored in the TCA-precipitable material in the medium.

3.1.9. The Effect of 2-Deoxyglucose on the Intracellular Incorporation of $[^3H]$-Glucosamine and $[^3H]$-Leucine after a 17 h Incubation Period

At the end of a 17 h incubation period, 2-deoxyglucose was seen

TABLE 3

The Effect of 2-Deoxyglucose on the Time Course of $[^3H]$-Glucosamine Incorporation into BHK Cells Grown on Normal EFC 10 Medium

Labelling period (h)	0-1	2-3	5-6	6-7
-I (dpm)	1,760	1,810	1,455	1,190
+I "	725	565	570	370
% Inhibition	58	68	61	70

BHK cells (6×10^5) were seeded in normal EFC 10 medium (3 ml). After 18 h, 2-deoxyglucose (0.02M, final concentration) was introduced into the medium prior to a 1 h incubation with $[^3H]$-glucosamine (2 μCi). The radioactivity in the intracellular TCA precipitable fraction was measured as described previously.

TABLE 4

The Effect of 2-Deoxyglucose on the Intracellular Incorporation and Extracellular Secretion of $[^3H]$-Glucosamine

Inhibitor	$[^3H]$-glucosamine incorporated into:		$[^3H]$-glucosamine secreted into medium (dpm) (TCA-precipitable counts)
	TCA-precipitable material (dpm)	TCA-soluble material (dpm)	
-	16,353	17,620	2,549
+	4,797	7,104	2,205
% Inhibition	71	60	8

The experiment was carried out as described in section 3.1.1. 2-Deoxyglucose (final concentration 0.02M) was added to the growth medium for 1 h prior to a 3 h incubation with $[^3H]$-glucosamine (2 μCi).

(Table 5) to inhibit incorporation of [^{3}H]-glucosamine into the intracellular acid-precipitable fraction by 82%. Incorporation of [^{3}H]-leucine was similarly inhibited by 63%.

3.1.10. The Effect of 2-Deoxyglucose on the Time Course of Incorporation of [^{3}H]-Fucose and [^{3}H]-Leucine into Intracellular Glycoproteins

The incorporation of [^{3}H]-fucose was subject to inhibition by 2-deoxyglucose to a maximum of 35% at the end of 7 h (Table 6a). Although this inhibition was less than for [^{3}H]-glucosamine under the same conditions, it will be noted that the total incorporation of [^{3}H]-fucose was small. Inhibition of [^{3}H]-leucine incorporation was similar to other comparable experiments.

3.1.11. The Effect of 2-Deoxyglucose on the Intracellular Incorporation and Extracellular Secretion of [^{3}H]-Fucose

It will be noted from Table 7 that [^{3}H]-fucose incorporation is low and very much less than that of [^{3}H]-glucosamine for similar experiments. Nevertheless, deoxyglucose appeared to inhibit incorporation of [^{3}H]-fucose into both the TCA precipitable and TCA soluble fractions by 38% and 26% respectively at the end of a 1.5 h labelling period. A 2.5 h labelling period demonstrated a significant increase in inhibition of [^{3}H]-fucose incorporation to 61% and 58% respectively.

The presence of deoxyglucose did not affect the extracellular [^{3}H]-fucose during a 1.5 h labelling period, but did cause a 23% inhibition when the isotope was present in the medium for 2.5 h.

3.1.12. The Effect of 2-Deoxyglucose on Intracellular Incorporation and Extracellular Secretion of Labelled Glucosamine

The experiment was carried out as indicated in Table 8. All plates,

TABLE 5

The Effect of 2-Deoxyglucose on Intracellular Incorporation of [^{3}H]-Glucosamine and [^{3}H]-Leucine

Inhibitor	[^{3}H]-Glucosamine Incorporated (dpm)	[^{3}H]-Leucine Incorporated (dpm)
-	1,388	10,770
+	243	3,989
% Inhibition	82	63

The experiment was carried out as described in the legend for Table 1. Final 2-deoxyglucose concentration was 0.02M.

TABLE 6

The Effect of 2-Deoxyglucose on the Time Course of Incorporation of $[^3H]$-Fucose and $[^3H]$-Leucine into Intracellular Glycoproteins

a) Incorporation of $[^3H]$-Fucose

Labelling period (h)	1-2	2-3	5-6	6-7
-I (dpm)	199	490	256	203
+I (dpm)	208	486	251	133
% Inhibition	0	0	2	35

b) Incorporation of $[^3H]$-Leucine

Labelling period (h)	1-2	2-3	5-6	6-7
-I (dpm)	2,127	4,339	5,285	4,824
+I (dpm)	1,204	1,912	2,127	1,834
% Inhibition	43	56	60	62

The experiment was carried out as described in the legend for Fig. 4. The concentration of 2-deoxyglucose in the extracellular growth medium was 0.02M prior to labelling with $[^3H]$-fucose (2 μCi) and $[^3H]$-leucine (20 μCi) for 1 h.

TABLE 7

The Effect of 2-Deoxyglucose on the Intracellular Incorporation and Extracellular Secretion of $[^3H]$-Fucose

	TCA-precipitable (dpm)		TCA-soluble (dpm)		Medium (TCA-precipitable) (dpm)	
Labelling period (h)	1.5	2.5	1.5	2.5	1.5	2.5
-I	417	795	493	906	756	960
+I for 6 h	259	310	364	378	726	743
% Inhibition	38	61	26	58	4	23

The experiment was carried out as described in section 3.1.1. 2-Deoxyglucose (0.02M) was present in the growth medium for 6 h prior to a $1\frac{1}{2}$ h or $2\frac{1}{2}$ h incubation with $[^3H]$-fucose (2 μCi).

TABLE 8

The Effect of 2-Deoxyglucose on Intracellular Incorporation

Petri dish No.	30 min pre-label of cells with 5 μCi [^{3}H]-glucosamine	Cells washed 2 x new EFC 10 added	I added to medium	5 μCi [^{14}C]-glucosamine	Further 2 h incubation
1	●	●			●
2	●	●	●		●
3				●	●
4			●	●	●
5	●	●		●	●
6	●	●	●	●	●
7	●				
8	●		●		

BHK cells (5×10^{6}) were seeded in petri dishes (50 mm) in EFC 10, glucose-free and 50 mM in pyruvate (3 ml). After 18 h duplicate pairs of plates were labelled with [^{3}H]-glucosamine and [^{14}C]-glucosamine as shown, with and in the absence of 2-deoxyglucose (0.02M). Radioactivity in the three fractions was monitored as described in section 3.1.1.

and Extracellular Secretion of Labelled Glucosamine

Intracellular glucosamine				Extracellular Medium (dpm)	
TCA-precipitable (dpm)		TCA soluble (dpm)			
^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
5,038	-	5,816	-	931	-
4,855	-	6,972	-	839	-
-	7,566	-	20,942	-	5,049
-	652	-	5,177	-	5,589
5,147	7,904	6,216	23,094	1,250	6,195
5,214	784	7,941	4,990	1,354	6,049
2,206	-	16,344	-	3,785	-
1,946	-	19,364	-	3,050	-

with the exception of 3 and 4 were pre-labelled with [^{3}H]-glucosamine (5 μCi) for 30 min. At the end of this time all plates had the growth medium removed and collected and the cells were washed twice with pre-warmed (37°C) BSS/bicarbonate solution. The cells, with the exception of plate 7 then received fresh pre-warmed growth medium (3 ml). Deoxy-glucose (0.02M) was added to plate No. 8 immediately prior to the growth medium being collected (zero time control). Deoxyglucose was added to the fresh growth medium in plates 2, 4, 6 and incubated for further 2 h in the case of plate 2; and in the case of 4 and 6 incubated for a further 2 h after the cells had been labelled with [^{14}C]-glucosamine (5 μCi).

The growth medium in plates 1 and 5 was changed and plate No. 1 incubated for a further 2 h. Plate No. 3 was incubated for 2 h after [^{14}C]-glucosamine (5 μCi) was introduced into the new medium.

The results of this experiment are shown in Table 8. When the inhibitor was added to cells pre-labelled with [^{3}H]-glucosamine (plates 1 and 2), it had no effect on the pre-formed sugar pool, neither on the intracellular acid-precipitable pool (i.e. glycoprotein pool), nor the extracellular secreted glycoprotein.

Comparing plates 3 and 4, where there was no pre-labelling, it was seen that 2-deoxyglucose had a marked effect on the incorporation of [^{14}C]-labelled glucosamine into the intracellular pools (acid soluble and acid precipitable), but had no effect on extracellular secretion.

Considering plates 5 and 6, the inhibitor present in plate 6 had no effect on the pre-labelled (tritiated) intracellular glucosamine pools, since 2-deoxyglucose had been introduced at the end of the pre-labelling period. It did , however, have an enormous inhibitory effect on the incorporation of the [^{14}C]-labelled glucosamine, where incor-poration (into TCA precipitable material) was reduced by a factor

of ten in the presence of 2-deoxyglucose and by a factor of five into the TCA soluble pool.

Plates 7 and 8 demonstrate the amount of $[^3H]$-glucosamine incorporation into each fraction at the end of a 30 min incubation period. Inhibitor is added to plate 8 at the end of this period immediately prior to removal of the extracellular medium and TCA precipitation i.e. plate 8 constitutes a zero time control.

3.1.13. An Investigation into the Effect of 2-Deoxyglucose on the $[^3H]$-Glucosamine-Labelled Material in the Extracellular Medium of Growing BHK Cells; and into the Nature of this Extracellular Material

Two Roux bottles were each seeded with BHK cells (5×10^6) in EFC 10 medium, glucose-free and 50 mM in pyruvate (50 ml). After 18 h the cells in both bottles were washed twice in pre-warmed (37°C) BSS and bicarbonate in order to remove traces of the growth medium.

Eagles medium which had been prepared without serum was pre-warmed to 37° and 50 ml added to each Roux. Deoxyglucose (0.02M) was present in one Roux prior to both being labelled with $[^3H]$-glucosamine (250 μCi) for 6 h. At the end of this incubation time the serum-free medium from each Roux was removed, dialysed extensively (for 3 days at 4°C) against 10 mM Tris buffer, pH 8.2, containing 0.15M KCl to remove excess free glucosamine. The dialysing buffer was changed regularly throughout the day.

The dialysed samples were lyophilised, re-dissolved in a small volume of buffer (1 ml) and loaded onto a Sephadex G50 column eluted with the same buffer. The profiles obtained are shown in Fig. 7(a) and (b).

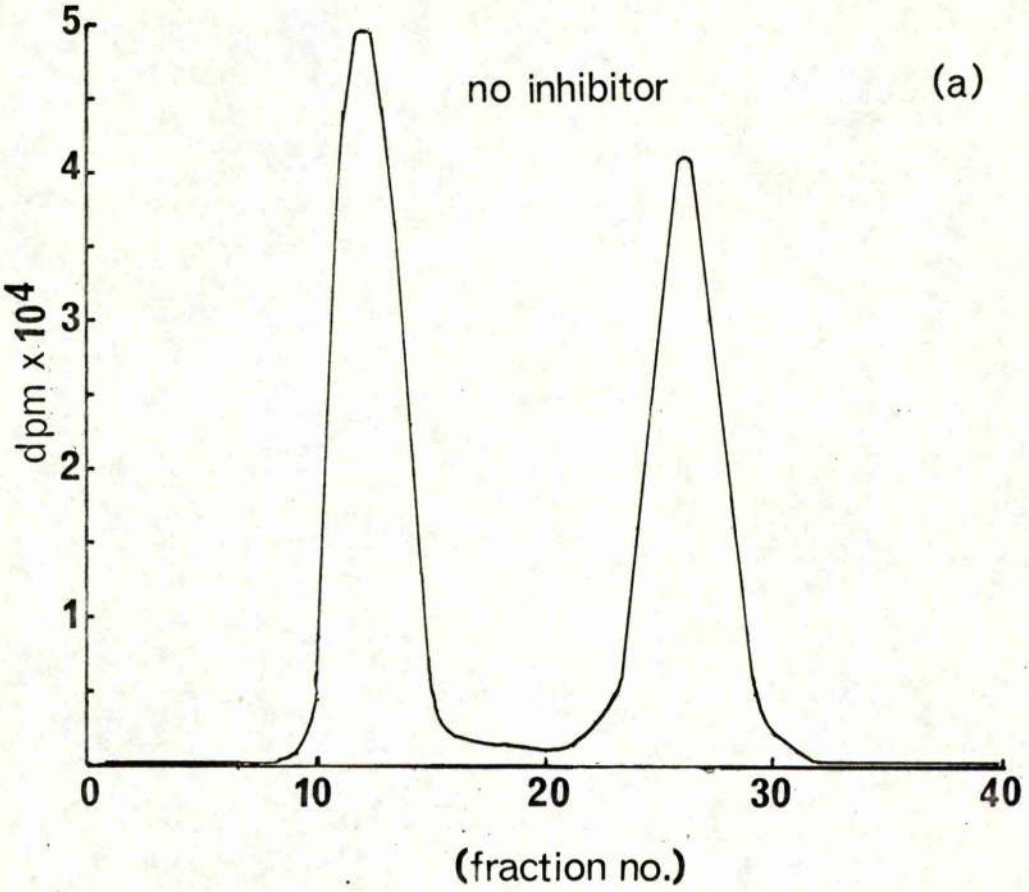
Those fractions which appeared from Fig. 7(a) to be eluted at the void volume of the column (i.e. the high molecular weight species) were

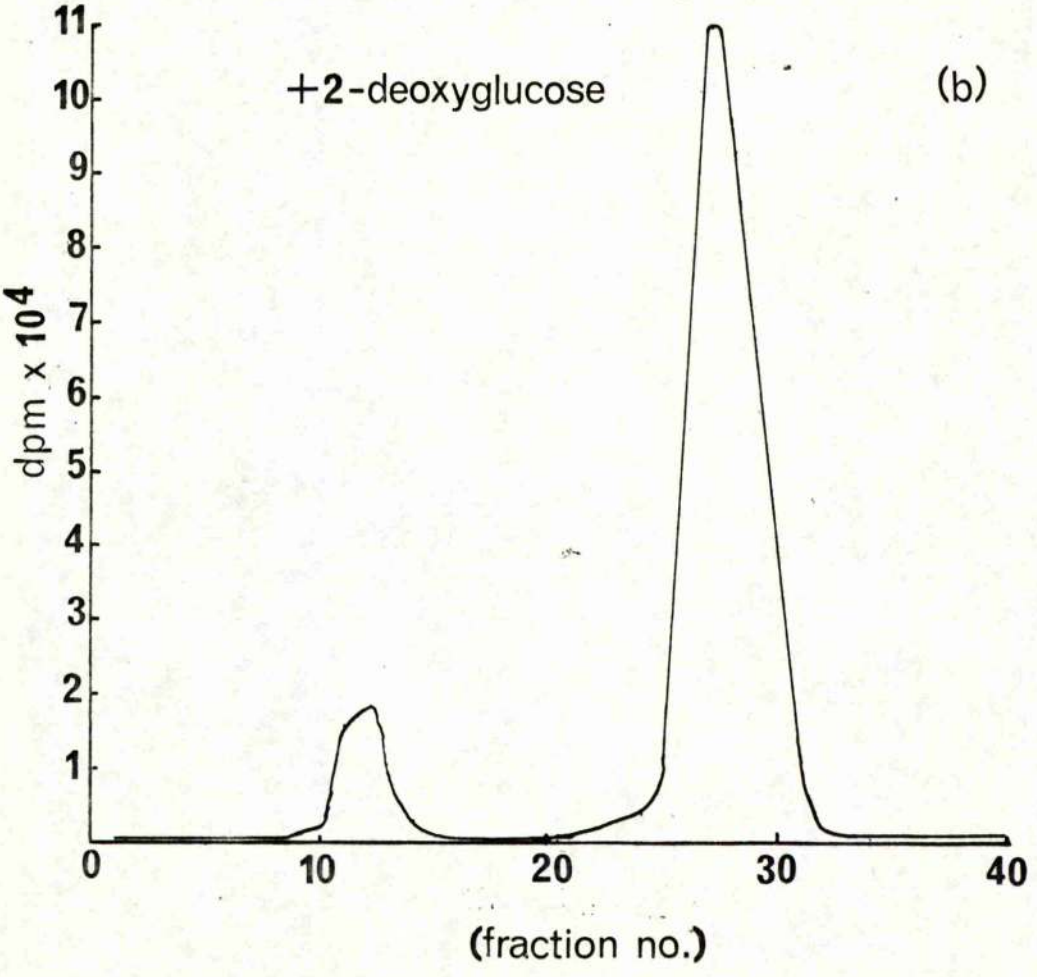
FIGURE 7 An Investigation into the Effect of 2-Deoxyglucose on the $[^3H]$-Glucosamine-Labelled Material in the Extracellular Medium of Growing BHK cells

Serum-free Eagle's medium (50 ml) collected from BHK cells labelled with $[^3H]$-glucosamine (250 μCi) was dialysed and freeze-dried as described in section 3.1.13. The sample was loaded onto a Sephadex G50 column (24.0 x 1.5 cm) and eluted with 10 mM Tris buffer, pH 8.2, containing 0.15M KCl at an elution rate of 11.6 ml h^{-1} cm^{-2}. Fractions (1.2 ml) were collected and aliquots (400 μl) taken for radioactive scintillation counting.

(a) shows the column profile obtained

(b) depicts the profile obtained when the cells were incubated with $[^3H]$-glucosamine in the presence of 2-deoxyglucose (0.02M)


no inhibitor
(a)
dpm x 10^4
5
4
3
2
1
0
10
20
30
40
(fraction no.)


+2-deoxyglucose
(b)
dpm x 10^4
11
10
9
8
7
6
5
4
3
2
1
0
10
20
30
40
(fraction no.)

pooled and divided into three parts of equal volume. Each was freeze dried and redissolved in a small volume (1 ml) of buffer. One sample was a control and was loaded, untreated, onto the same column of Sephadex G50. Of the remaining two samples, one was pre-treated with pronase (1 mg ml^{-1}) and the other with the enzyme hyaluronidase (1 mg ml^{-1}) prior to analysis on Sephadex G50. Figs. 7(a) and (b) demonstrate the effect which the presence of 2-deoxyglucose had on glycosylated material secreted into the extracellular medium. There was a 61% reduction in the high molecular weight material which was to be found isotopically labelled in the extracellular medium.

This was in striking contrast to results previously obtained in other experiments with 2-deoxyglucose (section 3.1.12.), when the inhibitor appeared not to influence $[^3H]$-glucosamine secretion.

Some attempt was made to elucidate the nature of this extracellular high molecular weight material. In comparing the pronase-digested sample, Fig. 8(a), with the control, Fig. 8(b), on Sephadex G50, it will be observed that the pronase had had little effect on the elution profile, indicating that the material was largely non-protein in nature. The hyaluronidase treated sample, however, Fig. 8(c), showed quite a shift in the elution pattern resulting from the cleavage of hyaluronidase specific bonds in this high molecular weight material.

Recoveries of tritium from Sephadex G50 were routinely between 89-97% of that applied to the columns.

3.1.14. Further Investigation of the Nature of the High Molecular Weight $[^3H]$-Glucosamine-Labelled Extracellular Material

The high molecular weight material obtained from a G50 separation as described in section 3.1.13. was lyophilised and redissolved in 0.01M ammonium acetate (2 ml) prior to pronase digestion (1 mg ml^{-1} incubated at 37°C for 2 h). The sample was subsequently applied to a DEAE-

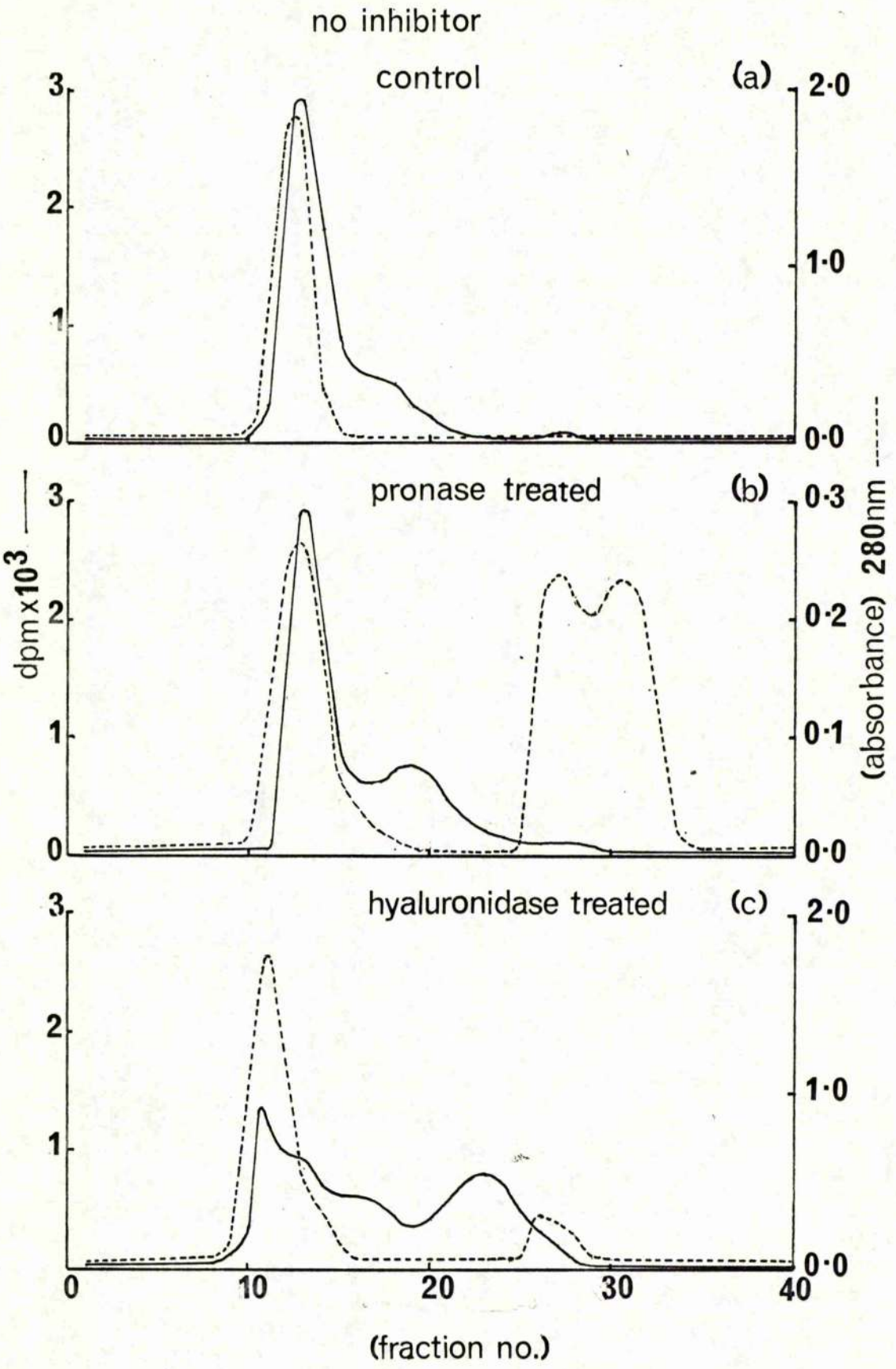
FIGURE 8 Gel Filtration on Sephadex G50 of Enzyme Digests of the High Molecular Weight Extracellular Material Eluted at the Void Volume in Fig. 7(a)

(a) Control

(b) Pronase digestion

(c) Hyaluronidase digestion

Enzyme concentrations were 1 mg ml^{-1} and the digestions were performed at 37°C for 2 h. Gel filtration was carried out as described in the caption for Fig. 7.


no inhibitor
control
(a)
pronase treated
(b)
hyaluronidase treated
(c)
dpm x 10³
(absorbance) 280nm
(fraction no.)
0
10
20
30
40
0·0
0·1
0·2
0·3
1·0
2·0
1
2
3

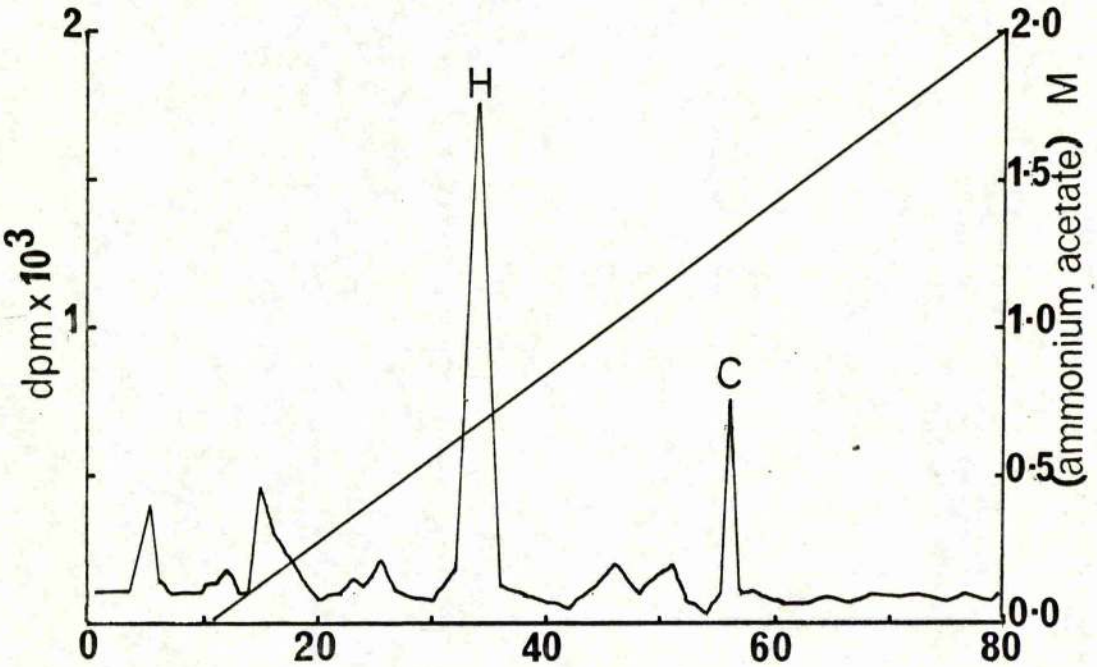
cellulose column in 0.01M $CH_3COO.NH_4$ and then eluted using a linear gradient of $CH_3COO\ NH_4$ between 0.01M and 2M (section 2.2.2.2.).

Two radioactive peaks were eluted (Fig. 9); one at a $CH_3COO\ NH_4$ concentration of 0.6M and the other eluted at a salt concentration of between 1.0M and 1.5M.

The nature of these two peaks will be discussed later in this dissertation.

FIGURE 9 Investigation of the High Molecular Weight Extracellular Material by DEAE-Cellulose Chromatography

High molecular weight material obtained from gel filtration on Sephadex G50 (Fig. 7a) was digested with pronase (1 mg (enzyme) ml^{-1} at 37°C for 2 h) and eluted from a DEAE-cellulose column by a linear gradient of ammonium acetate (0.01M - 2M). Fractions (5 ml) were collected and aliquots (1 ml) taken for counting.



(fraction no.)

H– hyaluronic acid

C– chondroitin sulphate

3.2. Solubility Studies

3.2.1. A Comparison of BHK C13 Nuclei by SDS Polyacrylamide Gel Electrophoresis Prepared by Two Methods and the Distribution Therein of Different Radioactive Isotopes

BHK C13 nuclei labelled with [^{3}H]-glucosamine were prepared by two methods, one using the non-ionic detergent Nonidet P40 (section 2.2.4.1.) and the other by a procedure not involving a detergent (2.2.4.2.). The resulting nuclear pellet from each preparation was subjected to SDS polyacrylamide gel electrophoresis and the gels sliced (2.2.2.3.) and monitored for the presence of labelled glycoproteins and glycolipids. A high proportion (92.0%) of the labelled material applied to the gels originally was accounted for in the labelled protein distribution pattern.

Fig. 10 illustrates the difference in profiles obtained as a result of the method chosen. The tris-sucrose preparation clearly shows the [^{3}H]-glucosamine distribution in the gel as well defined peaks of radioactivity.

Nuclei washed with Nonidet P40, on the other hand, gave a labelling profile in which the major carbohydrate-containing peaks were absent. Only 38.0% of the radioactive material present in the original nuclear fractions could be accounted for. Washing the nuclear pellet with Nonidet P40 appeared to have removed more than 60.0% of the [^{3}H]-glucosamine labelled components from the pellet.

It is notable that there was a major labelled component on the gel present at the dye front in the tris-sucrose preparation (Fig. 10). These [^{3}H]-labelled molecules correspond to glycolipid which was quantitatively extracted by Nonidet P40, thereby indicating the removal of the nuclear envelope by the detergent.

A photograph of coomassie blue stained gels from each nuclear

FIGURE 10 A Comparison by SDS Polyacrylamide Gel Electrophoresis of BHK C13 Nuclei Labelled with $[^3H]$-Glucosamine Prepared by Two Methods

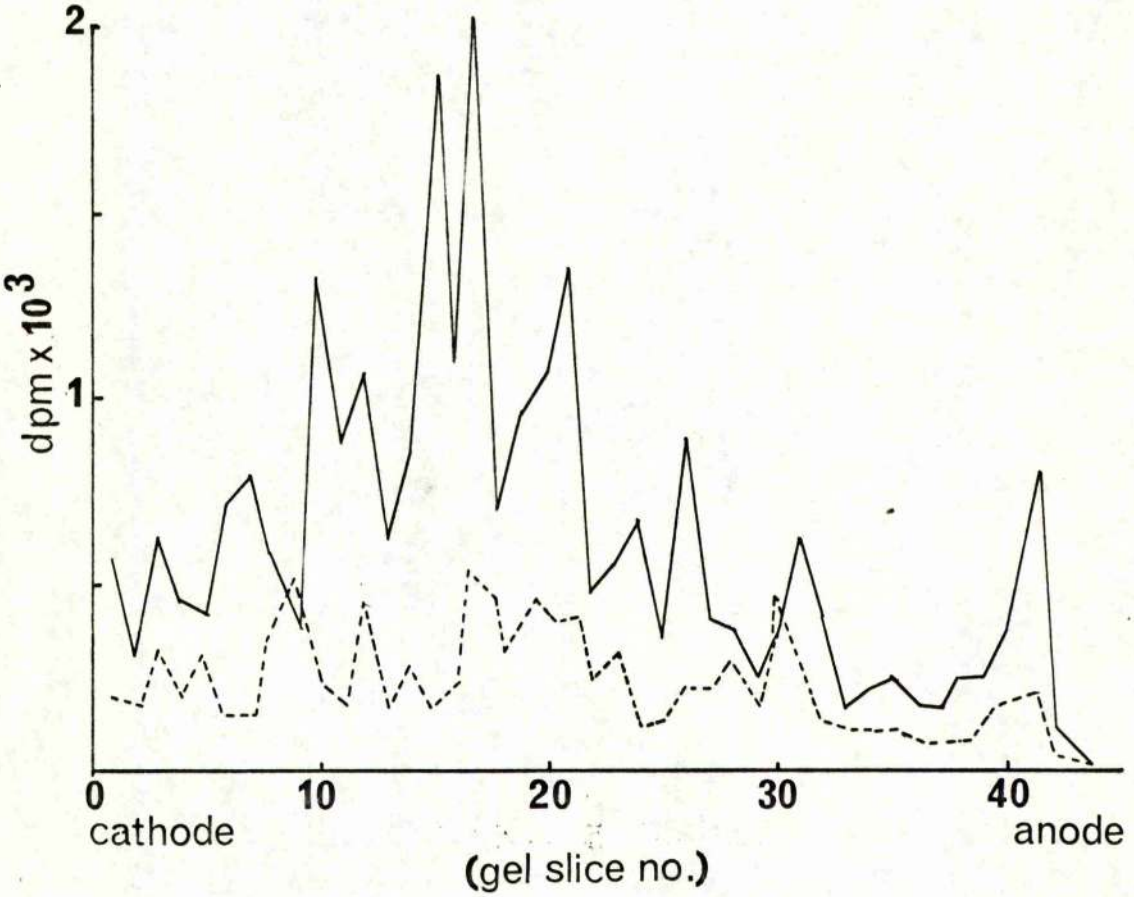
Two Roux bottles of BHK C13 cells were seeded each with 5×10^6 cells in normal EFC 10 (50 ml) and labelled with $[^3H]$-glucosamine (100 μCi) for 16 h. Nuclei were prepared by a method involving the non-ionic detergent Nonidet P40 (described in section 2.2.4.1.) and by a second method without detergent (2.2.4.2.).

The nuclear pellet from each preparation was subjected to SDS polyacrylamide gel electrophoresis and the gels (5.6%) were sliced (section 2.2.2.3.) and monitored for radioactivity (2.2.3.3.).

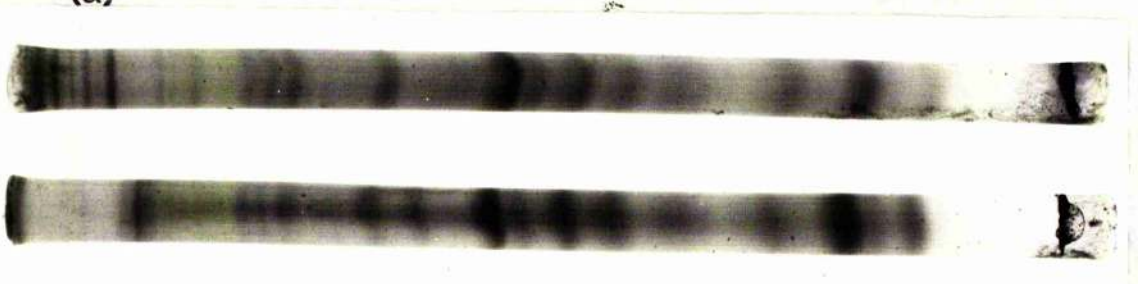
$[^3H]$-glucosamine from detergent made nuclei: --------

$[^3H]$-glucosamine from tris-sucrose prepared nuclei: ————

Photographs of coomassie blue stained gels are also depicted. Gel (a) illustrates the stained protein bands from nuclei prepared without detergent action and (b) those bands after Nonidet P40 treatment.



(a)



(b)

preparation is shown in Fig. 10, from which it will be seen that protein bands present at the high molecular weight end of the gel are absent from the Nonidet P40 treated nuclear protein pattern. This apart, the coomassie blue protein patterns on each gel from each preparation are similar, in spite of the drastic action of Nonidet P40 on the $[^3H]$-glucosamine labelled components mentioned earlier.

Nonidet P40 treatment of nuclei removes the $[^3H]$-labelled glycoprotein components of the nuclei. This detergent is known to disrupt membrane systems. Thus, from the gel shown in Fig.10, it may be inferred that the bulk of the nuclear proteins are not in the nuclear membrane and that the glycoprotein and protein components removed by Nonidet P40 form a minor proportion of the total nuclear protein.

Cl3 Nuclei prepared by the tris-sucrose procedure with $[^3H]$-glucosamine (Fig. 11(a)) and $[^3H]$-fucose (Fig. 11(b)) were also compared by SDS gel electrophoresis. Fucose is a sugar which is found specifically, though not always present, in glycoprotein and glycolipid. It was intended in future experiments concerning nuclear glyco-substances that $[^3H]$-glucosamine would be used to follow the incorporation of radioactivity into glycoprotein and glycolipid. It was, therefore, important to confirm at this stage that its incorporation profile did not differ substantially from that of $[^3H]$-fucose. Fig. 11 clearly illustrates that although the incorporation of $[^3H]$-fucose is lower than for $[^3H]$-glucosamine, the latter is not being randomly incorporated intracellularly, but is being used specifically in glycoprotein biosynthesis.

The finding (Fig. 10) that the non-ionic detergent Nonidet P40 removed the bulk of $[^3H]$-glucosamine labelled components from Cl3 nuclei suggested the possibility that these procedures selectively removed the glycoprotein/glycolipid associated with the nuclear envelope. In a further investigation nuclei from Cl3 cells were washed with a

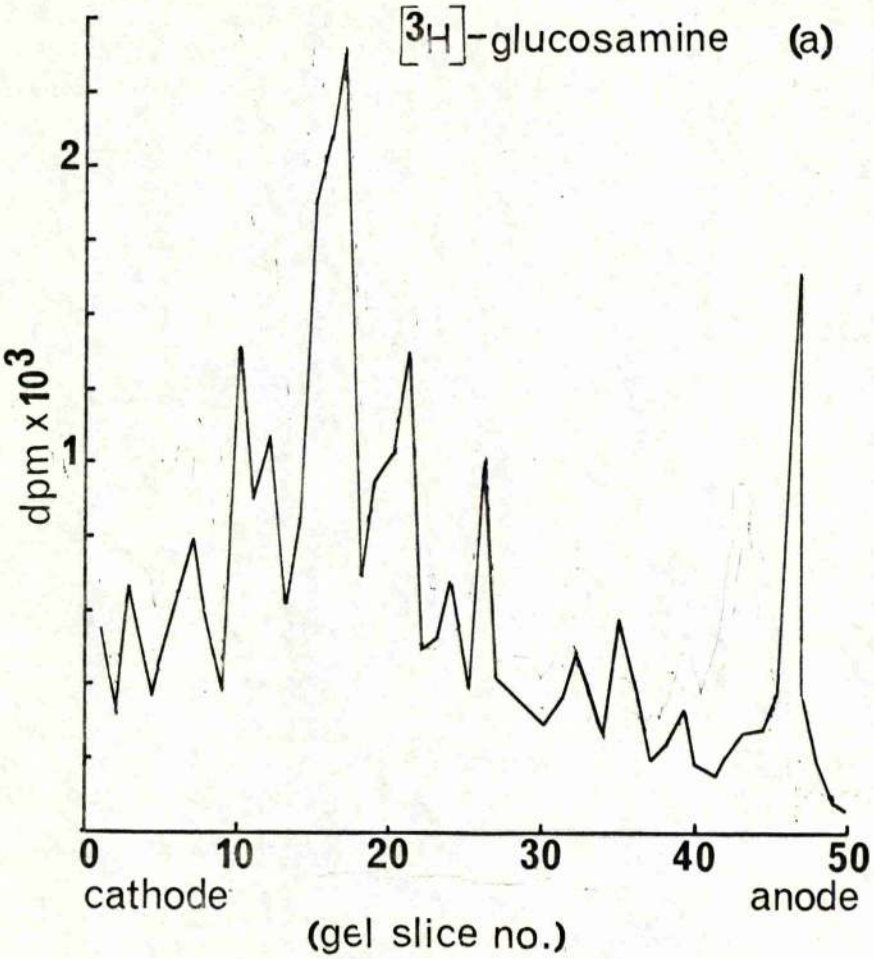
FIGURE 11 A Comparison by SDS Polyacrylamide Gel Electrophoresis of the Distribution of $[^3H]$-Glucosamine and $[^3H]$-Fucose in BHK Cl3 Nuclei

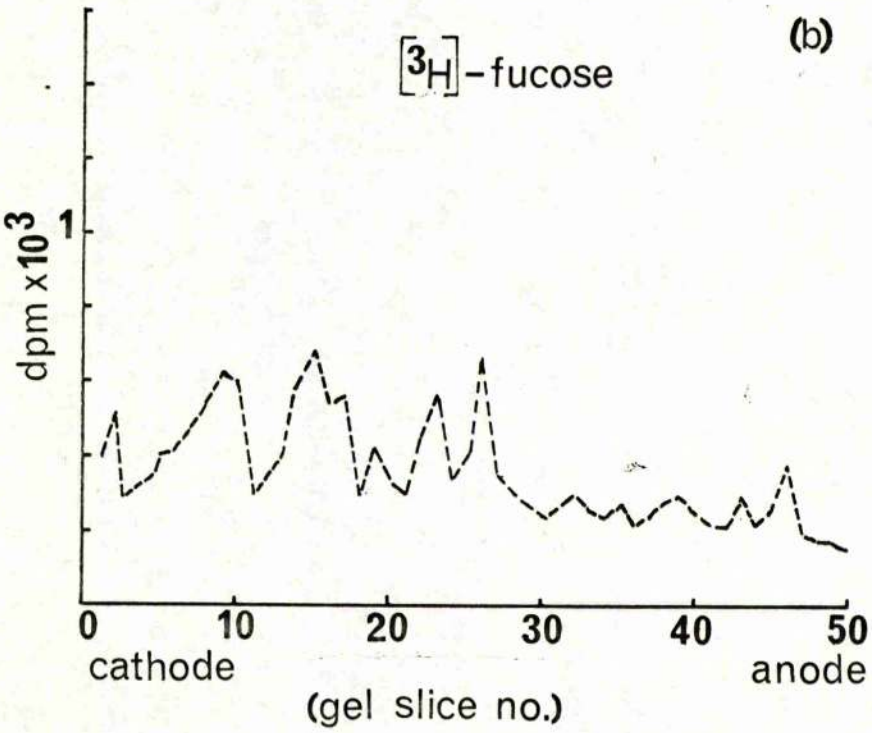
BHK Cl3 cells were seeded as described for Fig. 10 and labelled with $[^3H]$-fucose (100 μCi) and $[^3H]$-glucosamine (100 μCi) for 16 h.

Nuclei were prepared by the tris-sucrose method and the resulting (Fig. 11) radioactivity distribution profiles obtained as described for Fig. 10.

11a) Distribution of $[^3H]$-glucosamine: ————

11b) Distribution of $[^3H]$-fucose: --------


[3H]-glucosamine
(a)
dpm x10^3
2
1
0
10
20
30
40
50
cathode
anode
(gel slice no.)


[3H]-fucose
(b)
dpm x10^3
1
0
10
20
30
40
50
cathode
anode
(gel slice no.)

variety of non-ionic detergents (Tween 80, Triton X-100 and Nonidet P40) in order to investigate their relative abilities to solubilise carbohydrate containing components.

3.2.2. The Distribution of [^{3}H]-Glucosamine and [^{14}C]-Protein Label in Extracted Fractions of BHK C13 Nuclei

Labelled C13 nuclei prepared without the use of detergent (section 2.2.4.2.) were extracted sequentially in Tween 80, Triton X-100, Nonidet P40, 0.35M NaCl and finally in 1% ($^{w}/_{w}$) SDS. Table 9(a) shows that a small quantity of labelled material is removed in the first washing with Tween 80 (6.6% [^{3}H]-glucosamine and 5.8% [^{14}C]-labelled protein). Extraction with Triton X-100 removed the bulk of [^{3}H]-glucosamine (55.5%) and 38.0% of the labelled protein from the nuclei, resulting in only a small increase in the specific activity of the extracted glycoprotein fraction. This observation is in contrast to similar studies on BSC-1 cells, where preferential removal of the nuclear envelope is achieved, resulting in solubilisation of 80.0% of membrane associated glycoproteins and about 20-25% of the nuclear protein (Lindsay, J.G., Eason, R. and Adams, R.L.P., unpublished results). It seems likely that Triton X-100, in this instance, also permits release of a considerable amount of intranuclear protein and this is confirmed by comparison of results in Table 9(a) and 9(b). These tables demonstrate that prior extraction of the nuclei with 0.35M salt, a procedure routinely employed to remove nucleoplasmic and chromosomally-associated proteins, significantly decreases the fraction of nuclear protein which is subsequently susceptible to extraction by Triton X-100. Thus, BHK C13 cells appear to contain a class of proteins which are mutually released by either Triton X-100 or 0.35M NaCl.

Subsequent extraction of the nuclei with Nonidet P40 removed a further small percentage of labelled carbohydrate and protein (Table

TABLE 9

Two roller bottles were each seeded with BHK C13 cells (30×10^6) in glucose-free EFC medium with added pyruvate (50 mM). The cells in each bottle were labelled with $[^3H]$-glucosamine (100 μCi) and $[^{14}C]$-protein hydrolysate (5 μCi) for 16 h. Nuclei were prepared by the tris-sucrose method (section 2.2.4.2.).

The detergent solutions used in the sequential extraction tabulated on the adjacent page were all made up in tris-sucrose solution pH 7.5.

The nuclear pellet was suspended successively in each solution (1 ml) in the order shown in Table 9(a) and (b) and incubated for 10 min on ice before centrifugation at 1000xg (av.) for 10 min at 4°C.

The supernatant from each extraction was collected and monitored for radioactive material (section 2.2.3.1.).

TABLE 9

The Distribution of [^{3}H]-Glucosamine and [^{14}C]-Protein Label in Extracted Fractions of BHK C13 Nuclei

	% Extracted	
(a)	[^{3}H]-Glucosamine	[^{14}C]-Protein
1% ($^{v}/_{v}$) Tween 80	6.6	5.8
1% ($^{v}/_{v}$) Triton X-100	55.5	38.0
1% ($^{v}/_{v}$) Nonidet P40	11.2	14.2
0.35M NaCl	12.2	22.3
1% ($^{w}/_{v}$) SDS	14.5	19.1
(b)		
1% ($^{v}/_{v}$) Tween 80	5.1	5.6
0.35M NaCl	23.0	35.0
1% ($^{v}/_{v}$) Triton X-100	47.0	32.0
1% Nonidet P40	14.0	5.6
1% ($^{w}/_{v}$) SDS	11.0	22.0

9(a) and 9(b)), although it was thought possible that those counts could be due to Triton X-100 soluble material remaining trapped in the pellet of the 1000xg (av.) centrifugation following the Triton X-100 treatment. Further experiments (section 3.2.3.) were carried out in such a way (i.e. centrifugation at 100,000xg (av.) for 30 min following each extraction) that a hard-packed pellet was obtained at each step.

3.2.3. The Distribution of $[^3H]$-Glucosamine, $[^3H]$-Ethanolamine and $[^{14}C]$-Protein Labelled Components after Extraction with Membrane Perturbants followed by High Speed Centrifugation

The labelled C13 nuclei were sequentially extracted in 2.5% ($^w/_v$) citric acid; 1% ($^v/_v$) Triton X-100, 1% ($^v/_v$) Nonidet P40 (or vice-versa) and finally in 1% ($^w/_v$) SDS as described in the legend for Table 10. Each solubilised extract was subjected to centrifugation at 100,000xg (av.) for 30 min to ensure that components in the supernatant liquid were truly soluble and that such procedures were not merely removing particulate material e.g. ribosomes or contaminating membranes which may be adhering to the surface of the nuclear envelope. Table 10(a) and (b) reveals that under such conditions citric acid will remove $[^{14}C]$-labelled protein (45.0%) and negligible amounts of labelled lipid and carbohydrate (3.4% and 8.0% respectively - such levels are considered insignificant because the results of such experiments have been found to be accurate only within 5.0%).

When the $[^3H]$-ethanolamine labelled C13 nuclei were subsequently extracted in Triton X-100 (Table 10(b)) prior to Nonidet P40 treatment, 89.0% of the labelled nuclear lipid was removed in the first instance, but 6.9% remained to be extracted by Nonidet P40.

When nuclei labelled with $[^{14}C]$-protein and $[^3H]$-glucosamine are

TABLE 10

Two roller bottles of BHK C13 nuclei were seeded as for Table 9. One such bottle was labelled with $[^{3}H]$-glucosamine (100 μCi) and $[^{14}C]$-protein hydrolysate (5 μCi) for 16 h, while the other was labelled with $[^{3}H]$-ethanolamine (10 μCi).

Nuclei prepared by the tris-sucrose method (2.2.4.1.) were extracted initially in 2.5% ($^{w}/_{v}$) citric acid; and successively in 1% ($^{v}/_{v}$) Triton X-100, 1% ($^{v}/_{v}$) Nonidet P40 (Table 10(a)), or vice-versa (Table 10(b)), and finally in 1% ($^{w}/_{v}$) SDS.

Each suspension was incubated on ice for 30 min and a pellet obtained by centrifugation at 100,000xg (av.) for 30 min at 4°C.

The supernatant in each case was monitored for radioactivity as previously described (2.2.3.1.).

TABLE 10

The Distribution of $[^{3}H]$-Glucosamine, $[^{3}H]$-Ethanolamine and $[^{14}C]$-Protein Labelled Components after Extraction with Membrane Perturbants followed by High Speed Centrifugation

(a)

	% Solubilised $[^{3}H]$-Glucosamine	% Solubilised $[^{14}C]$-Protein
2.5% ($^{w}/_{v}$) Citric acid	8.0	45.0
1% ($^{v}/_{v}$) Nonidet P40	80.0	28.0
1% ($^{v}/_{v}$) Triton X-100	8.9	9.4
1% ($^{w}/_{v}$) SDS	3.0	17.0

(b)

	$[^{3}H]$-Ethanolamine % Solubilised
2.5% ($^{w}/_{v}$) Citric acid	3.4
1% ($^{v}/_{v}$) Triton X-100	89.0
1% ($^{v}/_{v}$) Nonidet P40	6.9
1% ($^{w}/_{v}$) SDS	0.5

treated initially with Nonidet P40 and subsequently with Triton X-100 (Table 10(b)), 80.0% of the labelled carbohydrate and 28.0% of the protein is removed in the first instance, while Triton X-100 extracts a further 9.4% $[^{14}C]$-labelled protein and 8.9% tritiated glucosamine.

An interesting point to emerge from Table 10(a) and (b) is that while citrate treatment leads to extraction of 45.0% of the nuclear protein with little corresponding effect on the nuclear lipid or glycoprotein. Subsequent extraction with non-ionic detergents yielded 80.0% of the nuclear glycoprotein and 89.0% of the lipid, whilst 25-30% of the $[^{14}C]$-labelled protein was removed. Thus prior extraction with citric acid under the conditions specified appears to allow a more selective removal of the nuclear envelope fraction by non-ionic detergents.

3.2.4. The Release of Nuclear Membrane by Citric Acid

C13 Nuclei labelled with $[^{3}H]$-glucosamine and those labelled with $[^{3}H]$-ethanolamine were extracted in 2.5% ($^{w}/_{v}$) citric acid under conditions described by Smith et al. (1969) for the preparation of the outer nuclear envelope (legend for Table 11).

It was found (Table 11) that nuclear material labelled with either $[^{3}H]$-glucosamine or $[^{3}H]$-ethanolamine is removed by citric acid to the same extent (20%).

This contrasts markedly with the result shown in Table 10. The citrate effect on the nuclear membrane will be further investigated in section 3.3.

3.2.5. Further Investigation of the Effect of the Detergent Tween 80 on BHK C13 Nuclei Labelled with $[^{3}H]$-Glucosamine and $[^{14}C]$-Protein Hydrolysate

$[^{3}H]$-Glucosamine and $[^{14}C]$-protein labelled BHK C13 nuclei were subjected to 1% ($^{v}/_{v}$) Tween 80 under conditions described in the legend

TABLE 11

The Release of BHK C13 Nuclear Membrane by Citrate

	% Extracted from C13 Nuclei	
	$[^3H]$-Glucosamine	$[^3H]$-Ethanolamine
2.5% Citric acid	20%	20%

Note Citric acid extract centrifuged at 1,000xg (av.) for 10 min at 4°C.

BHK C13 cells seeded as for Table 9 were labelled with $[^3H]$-glucosamine (250 μCi) for 16 h. Cells were also grown in the same medium in a Roux bottle (5×10^6 cells) and labelled for the same period with $[^3H]$-ethanolamine (50 μCi).

The resulting nuclear pellets (section 2.2.4.2.) were each incubated with 2.5% ($^w/_v$) citric acid for 10 min on ice and centrifuged for 10 min at 4°C at 100,000xg (av.).

for Table 12.

In contrast to the result obtained from Table 9, it would appear that a 30 min incubation of the nuclear suspension on ice followed by high speed centrifugation causes removal of 17.0% of the $[^{3}H]$-glucosamine and 21% of the $[^{14}C]$-protein.

TABLE 12

The Effect of Tween 80 on BHK C13 Nuclei

	% Extracted from C13 Nuclei	
	$[^{3}H]$-Glucosamine	$[^{14}C]$-Protein
1% Tween 80	17%	21%

BHK cells were seeded as for Table 9 and labelled with $[^{3}H]$-glucosamine (250 μCi) for 16 h. C13 nuclei prepared by the tris-sucrose method (2.2.4.2.) were incubated in 1% ($^{v}/_{v}$) Tween 80 for 30 min on ice. The suspension was centrifuged at 100,000xg (av.) for 30 min at 4°C and the supernatant monitored for radioactivity (2.2.3.1.).

3.3. Isolation and Characterisation of the Nuclear Membrane from BHK Cell Lines

3.3.1. Isolation of Nuclear Membrane of BHK Cell Lines

Two methods of preparation were routinely employed in the isolation of nuclear membrane (section 2.2.5.1. and 2.2.5.2.). Both methods yield a crude nuclear membrane preparation which was further purified by centrifugation at 200,000xg (av.) for 16 h at 4°C on a sucrose gradient (Fig. 12(a)) in the following density steps: 1.22 g ml^{-1} (50% ($^{w}/_{w}$)), 1.20 g ml^{-1} (43% ($^{w}/_{w}$)) and 1.18 g ml^{-1} (37% ($^{w}/_{w}$)).

Material was seen to band at both the 1.22/1.20 g ml^{-1} sucrose interface (Band 1) and the 1.20/1.18 g ml^{-1} interface (Band 2) (Fig. 12(b)). In addition, a proportion of the crude membrane sample did not penetrate the gradient (Band 3), but had become different in appearance and texture to the well-suspended sample applied initially to the gradient. The material remaining at the top of the step-gradient appeared to form aggregates. This phenomenon was later found to be independent of the cell type (C13 or PYY) or the method of nuclear membrane preparation.

The density gradients were fractionated and the distribution of $[^{3}H]$-glucosamine was monitored. The elution profile of the label is seen in Figs. 13(a) and (b).

From Fig. 13(a), showing the distribution of $[^{3}H]$-glucosamine in nuclear prepared by the sonication method, it can be seen that 29.0% of the label applied to the gradient remained in Band 3, while the remainder is almost equally distributed between Band 1 (33.4%) and Band 2 (34.0%).

The distribution of $[^{3}H]$-glucosamine differs from the above when the nuclear membrane was isolated using the heparin method (Fig. 13(b)). A greater proportion of radioactive material was found in Band 1 (35.0%); than in Band 2 (27.0%) while 21.0% $[^{3}H]$-glucosamine remained in Band 3.

FIGURE 12 Isolation of Nuclear Membrane from BHK Cell Lines

BHK C13 cells and their polyoma transformed counterparts, PYY were seeded in Roller bottles at a concentration of 30×10^6 cells in glucose-free EFC 10 (180 ml) which was 50 mM in pyruvic acid. Each bottle of cells was labelled with $[^3H]$-glucosamine (1 mCi) for 16 h. Nuclei were prepared without the use of detergents (section 2.2.4.2.) and nuclear membrane prepared by two methods described in sections 2.2.5.1. and 2.2.5.2.

Fig. 12(a) illustrates the application of the crude nuclear membrane material to a step-wise sucrose gradient of 1.22 g ml^{-1} (50%, $^w/_w$), 1.20 g ml^{-1} (43%, $^w/_w$), 1.18 g ml^{-1} (37%, $^w/_w$). Following a 16 h centrifugation described in the methods section, three bands were observed, Fig. 12(b).

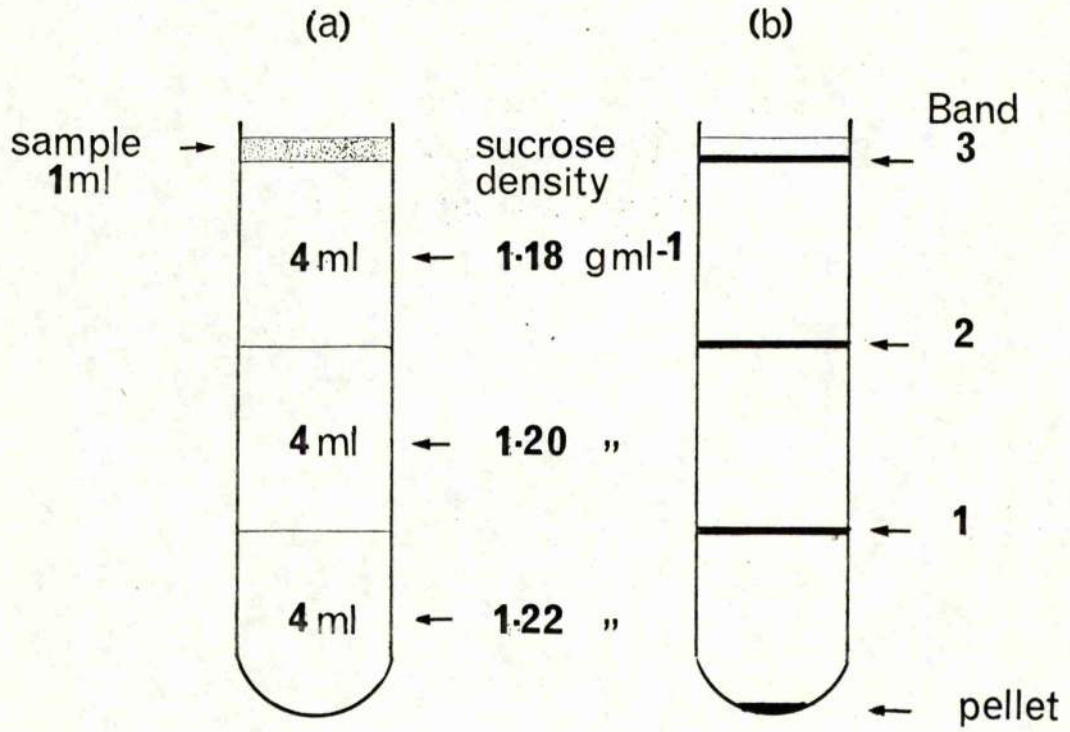
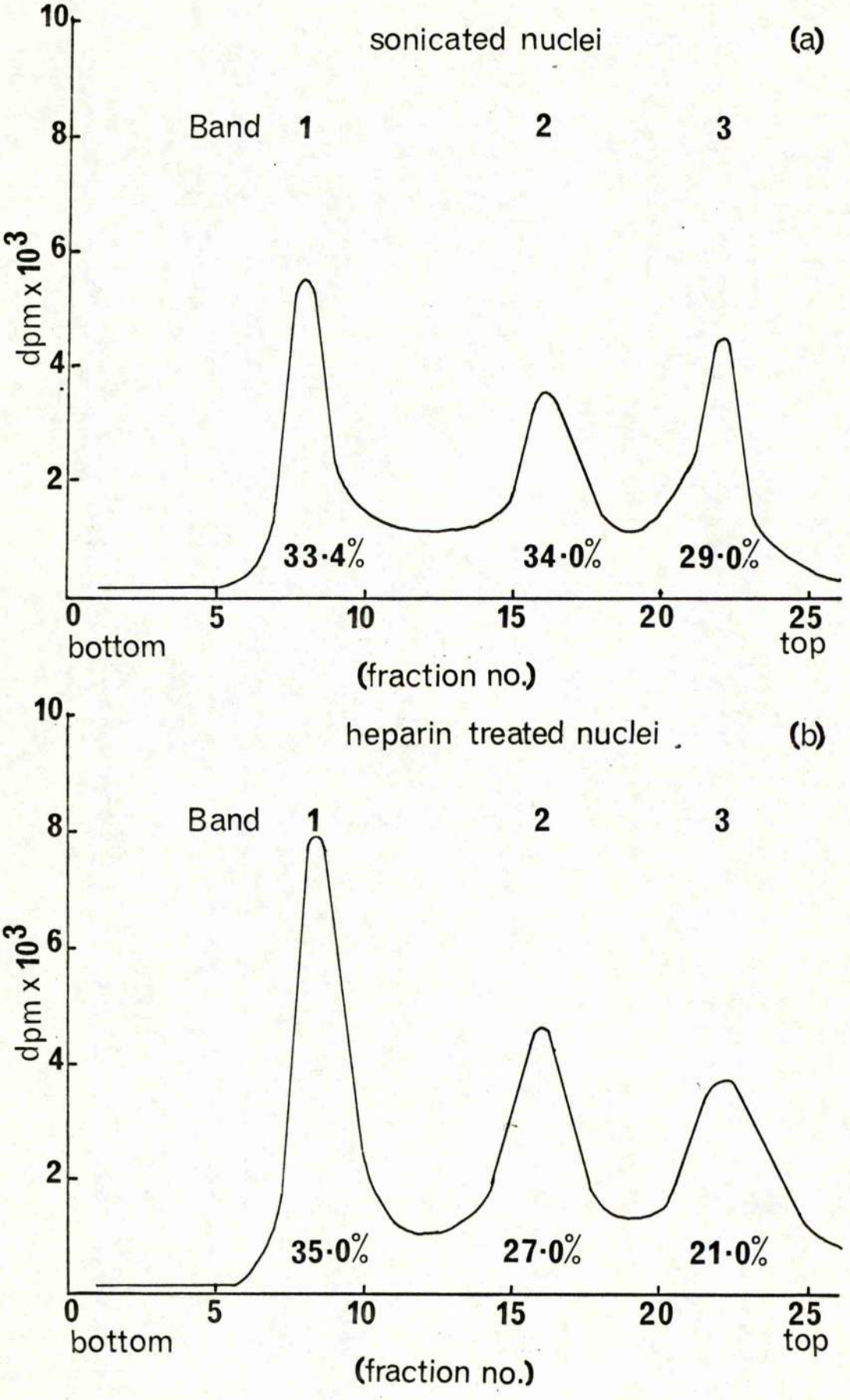

(a)
(b)
sample
1ml
sucrose
density
4ml
1·18 gml-1
4ml
1·20 „
4ml
1·22 „
Band
3
2
1
pellet

FIGURE 13 A Comparison of the Recovery of $[^3H]$-Glucosamine in BHK C13 Nuclear Membrane Preparations

Nuclear membrane was prepared from 2 roller bottles of BHK C13 cells as described previously in the legend for Fig. 12.

The gradient elution profile resulting from nuclear membrane prepared from the method detailed in section 2.2.5.1. (sonication) is shown in Fig. 13(a); and that from the heparin method (section 2.2.5.2.) is illustrated in Fig. 13(b).

The distribution of $[^3H]$-glucosamine in each of the 3 bands is shown


sonicated nuclei
(a)
Band 1
2
3
dpm x 10^3
10
8
6
4
2
33·4%
34·0%
29·0%
0
5
10
15
20
25
bottom
top
(fraction no.)
heparin treated nuclei
(b)
Band 1
2
3
dpm x 10^3
10
8
6
4
2
35·0%
27·0%
21·0%
0
5
10
15
20
25
bottom
top
(fraction no.)

Material from the Bands 1, 2 and 3 and the pellet was subjected to SDS polyacrylamide gel electrophoresis. Those heparin treated nuclear fractions prepared on sucrose gradients from Cl3 and PYY cells are depicted in Figure 14. The gels of these fractions were stained for the presence of protein with coomassie brilliant blue.

3.3.2. A Comparison of BHK Cl3 and PYY Nuclear Membrane on SDS Polyacrylamide Gel Electrophoresis

BHK Cl3 and PYY cells were grown under the conditions described for Figs. 15 and 16. Nuclear membrane was prepared using heparin (section 2.2.5.1.).

Bands 1-3 were observed for both types of BHK cells, as previously described for Cl3 cells. All six bands, as well as the very fine pellet to be found at the bottom of the gradient were washed and subjected to SDS polyacrylamide gel electrophoresis.

The distribution of the $[^3H]$-glucosamine in the gel slices is illustrated in Fig. 15 and 16. Fractionated Cl3 nuclear membrane from Bands 3, 2, 1 and the pelletted material are portrayed in Fig. 15(a), (b), (c) and (d) respectively. Similarly, Fig. 16(a)-(d) shows the corresponding PYY nuclear membrane fractions.

Estimates of the molecular weights of the major $[^3H]$-glucosamine labelled nuclear membrane components were obtained by comparison on polyacrylamide gels with the mobilities of standard proteins (Fig. 17) using the method of Carpenter and Harrington (1972).

Considering Fig. 15(a)-(d), it can be deduced that there are three major $[^3H]$-glucosamine labelled protein bands which are not only common to all three Cl3 membrane fractions, but also to the pelletted material. These fall into the molecular weight ranges of 110-100,000, 55-60,000 and 32-35,000 respectively. There are also three glycoproteins (molecular weights 74-84,000, 45-48,000 and 12-14,000) which

FIGURE 14 SDS Polyacrylamide Gel Electrophoresis of Nuclear Membrane Derived Fractions

These fractions of nuclear derived material from BHK C13 and PYY cells were taken from sucrose density gradients (viz. Pellet, Bands 1, 2 and 3) and were subjected to electrophoresis in SDS on 5.6% polyacrylamide gels and stained for protein with coomassie brilliant blue.

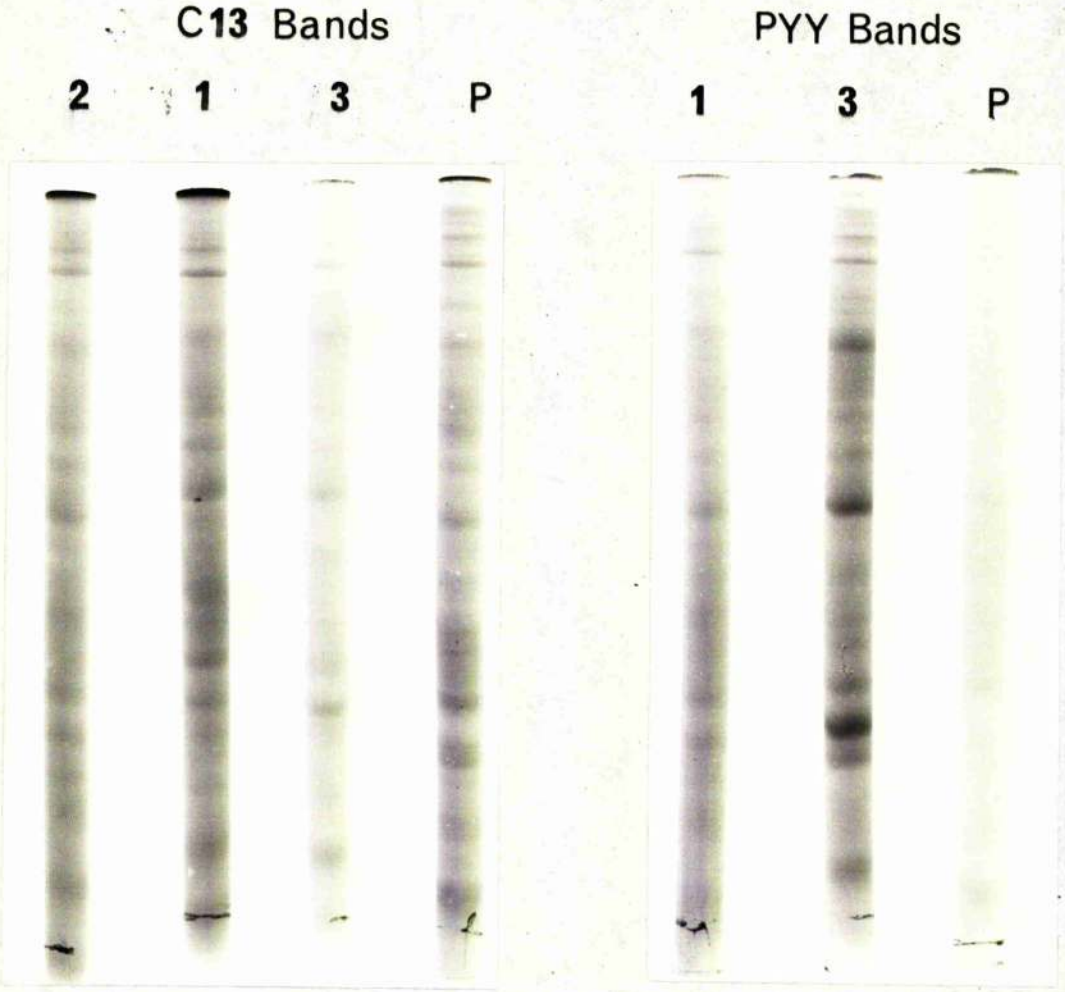

C13 Bands
2
1
3
P
PYY Bands
1
3
P

FIGURE 15 & 16 Characterisation of the Nuclear Membrane Bands of C13 and PYY Cells by Sodium Dodecyl Sulphate Gel Electrophoresis

Two roller bottles were seeded one with C13 cells and the other with PYY cells (section 2.2.1.) in glucose-free EFC 10 supplemented in this case with 50 mM pyruvate and 5 mM glucose. Each bottle was labelled with 1 mCi $[^3H]$-glucosamine for 16 h.

Nuclear membrane was prepared as in section 2.2.5.1. (using heparin). Gel electrophoresis was carried (2.2.2.3.) out on each band and on the pellet, the same amount of radioactivity being applied to each gel (approx. 30,000 dpm). The unstained gels were sliced (2.2.2.3.) and counted (2.2.3.3.). The resulting profiles for each band are shown in Figs. 15 and 16.

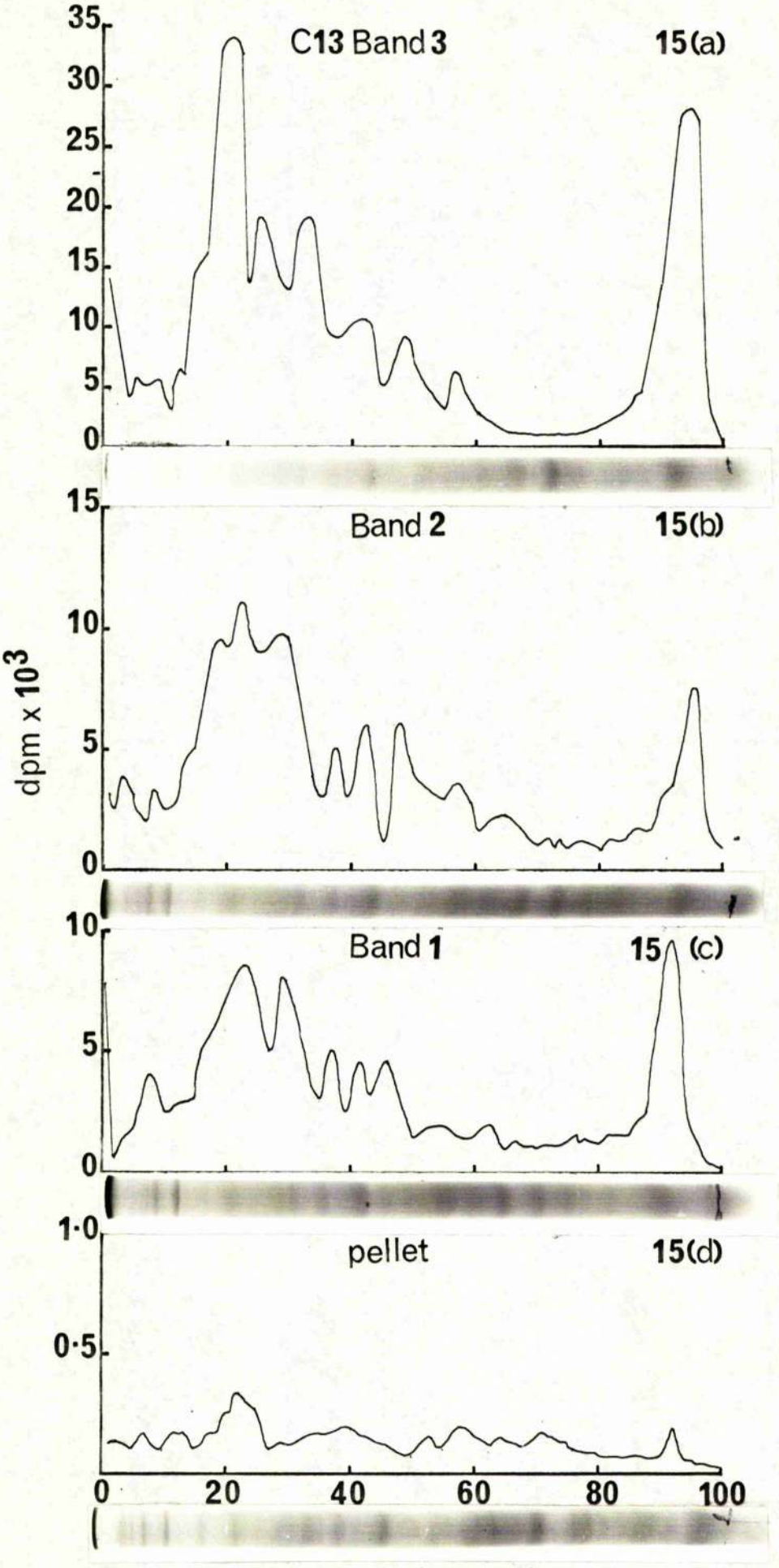
Fig. 15(a) C13 nuclear membrane band 3

(b) C13 nuclear membrane band 2

(c) C13 nuclear membrane band 1

(d) Material pelletted through the gradient

Fig. 16(a)-(d) Shows corresponding gel profiles for PYY cells


C13 Band 3
15(a)
Band 2
15(b)
Band 1
15 (c)
pellet
15(d)
dpm x 10^3
(gel slice no.)

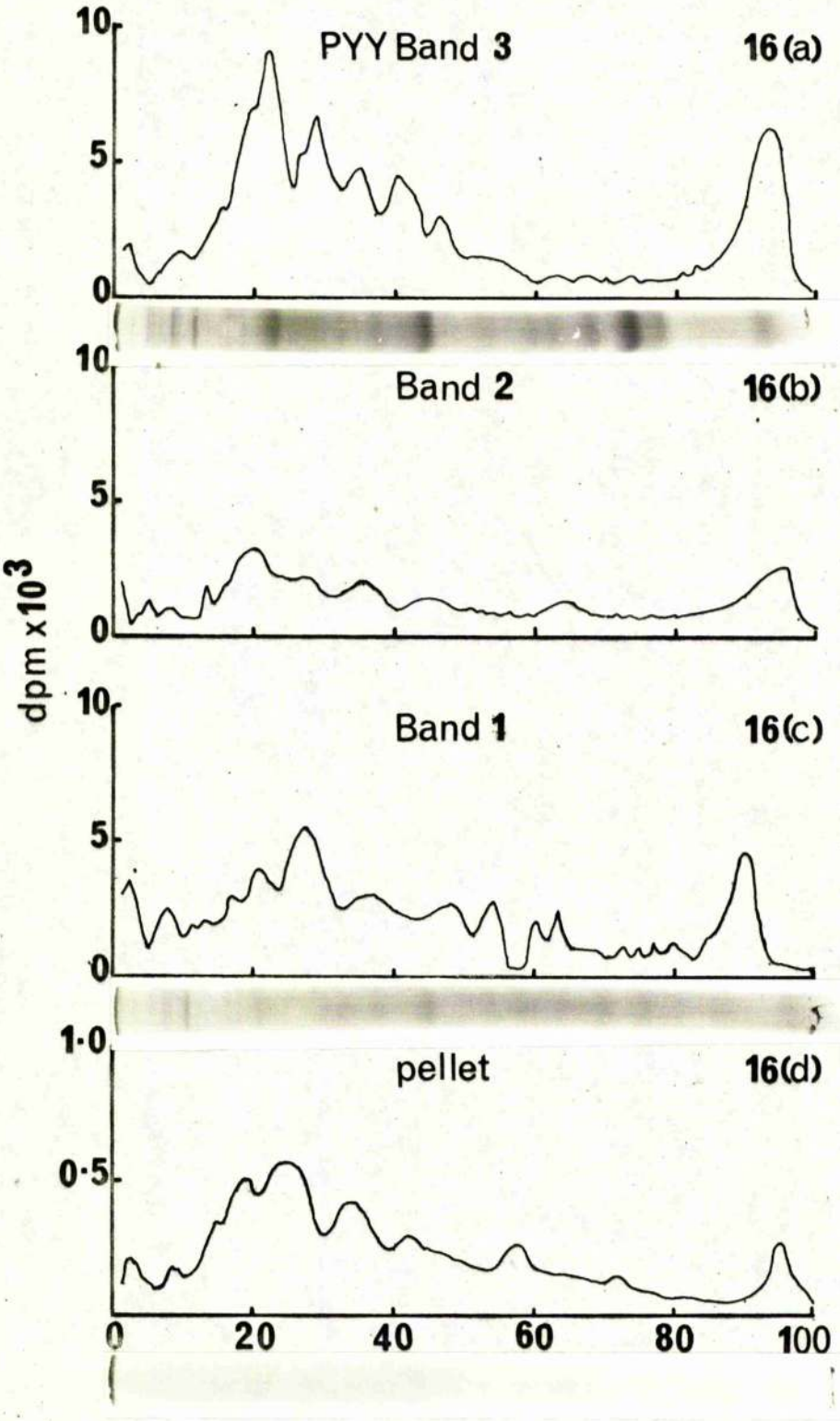
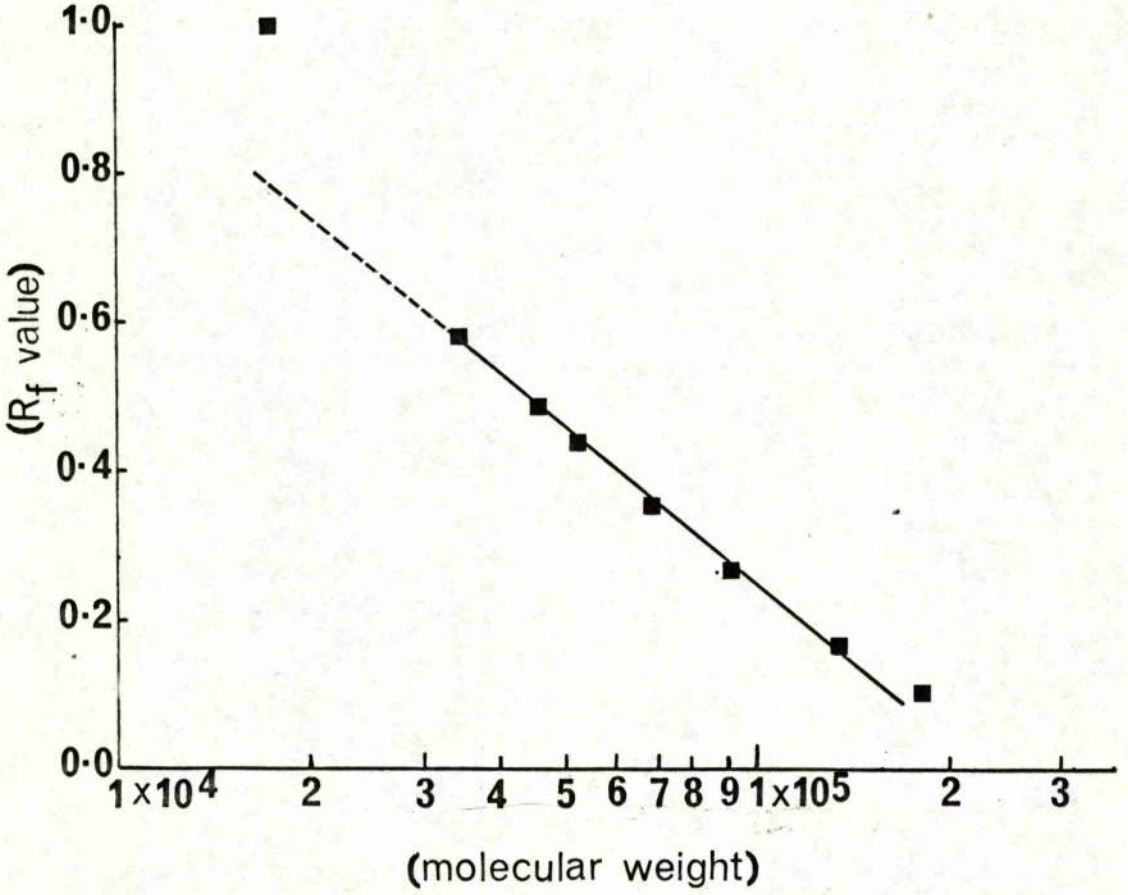

PYY Band 3
16(a)
Band 2
16(b)
Band 1
16(c)
pellet
16(d)
dpm x10³
10
5
0
1·0
0·5
0
20
40
60
80
100
(gel slice no.)

FIGURE 17 Molecular Weight Standard Graph

Proteins of known molecular weight, namely lysosyme, aldolase; ovalbumin and myoglobin and oligomers of these two (as described in the method of Carpenter and Harrington, 1972) were each applied to 5.6% polyacrylamide gels and subjected to electrophoresis simultaneously with the nuclear membrane components from BHK C13 and PYY cells isolated on sucrose density gradients.

Fig. 17 shows a logarithmic plot of MW versus the Rf value of each protein.


1·0
0·8
0·6
0·4
0·2
0·0
(R_f value)
1×10⁴
2
3
4
5
6
7
8
9
1×10⁵
2
3
(molecular weight)

are common to all three nuclear membrane bands, but which do not appear in the pellet. The smallest of these (12-14,000) coming at the low molecular weight end of the gel was considered to be glycolipid. Further, C13 Band 2 exhibits a glycoprotein of molecular weight 117,000 and C13 Band 3 are of molecular weight 94-95,000 which are specific to those bands.

Fig. 16(a)- (d) demonstrates that the PYY nuclear membrane bands 1, 2, 3 as well as the pellet have three high molecular weight glycoproteins in common. Their range of molecular weights is 105-114,000; 84-90,000 and 66-74,000. One protein band (55-60,000) appears only in the PYY Band 3 and the pellet; and another glycoprotein (molecular weight, 35,000) is peculiar to the PYY pellet. Glycolipid is present in all four fractions of both C13 and PYY nuclei.

In making a direct comparison of the glycoproteins of C13 and PYY nuclear membrane bands, three points can be made.

(i) Glycolipid was to be found in all nuclear membrane fractions for both cell lines.

(ii) Two high molecular weight glycoproteins (105-114,000 and 84-94,000) were common to all Bands 1, 2 and 3 from C13 and PYY cells.

(iii) Three low molecular weight glycoproteins (35,000, 45-46,000 and 55-60,000) were common to Bands 1, 2 and 3 of C13 cells, but were not present in any of the PYY nuclear membrane material.

3.3.3. Investigation of BHK Nuclear Membranes by Electron Microscopy

Electron micrographs were obtained (section 2.2.11.) from the nuclear membrane bands of BHK C13 and PYY cells. Those for Band 1 in each cell line are shown in Fig. 18(a) and (b).

The material appears membranous in nature. There is no evidence of the double nuclear membrane in these micrographs. It must be concluded, therefore, that the inner and outer nuclear membranes have been separated as, indeed, is borne out by the appearance of both rough membranes (containing ribosomes), and smooth membranes.

Furthermore, aggregates of nuclear pore complexes are also observed ('wheel-like' structures).

It would appear from morphological evidence that there are no detectable differences between Band 1 preparations from C13 and PYY cells.

3.3.4. Estimation of the Amount of Protein Present in BHK C13 and PYY Nuclear Membrane Components

Nuclear membrane prepared from BHK C13 and PYY cells by the use of heparin (section 2.2.5.1.) and membrane bands 1 and 2 from each cell line were assayed for protein (section 2.2.7.). The average result from several determinations which were carried out in duplicate is given in Table 13.

C13 Band 1 consistently the broadest of all the bands had, not surprisingly the most protein (110 μg approximately). C13 Band 2 contains approximately half the amount of protein found in Band 1 (55 μg).

By contrast, the most easily observable PYY nuclear membrane Band 1 contains 55 μg protein, and Band 2, which is not always observable, at best in the region of 45-50 μg protein. Material was only

FIGURE 18 Electron Micrographs of BHK C13 and PYY Nuclear Membrane

Electron micrographs of band 1 of C13 (Fig. 18(a)) and PYY (Fig. 18(b)) are demonstrated.

Magnification is 30,000x.

(a) C13 Band 1

(b) PYY Band 1

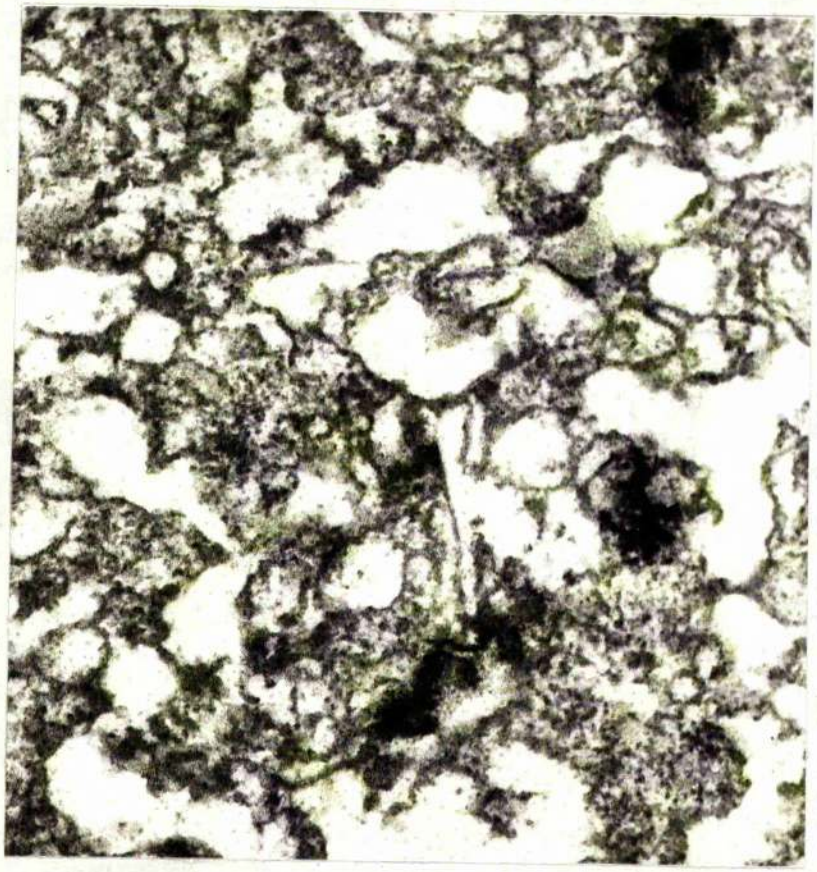


TABLE 13

Estimation of Protein in BHK C13 and

PYY Nuclear Membrane Bands

Sample	C13 (μg protein)	PYY
Band 1	110	55
Band 2	55	45

Nuclear membrane was prepared from BHK C13 and PYY cells (30×10^6) seeded in glucose-free EFC 10, 50 mM in pyruvate by the method in section 2.2.5.1. The C13 and PYY membrane material floating at both the 50/43% ($^{w}/_{w}$) sucrose interphase (Band 1) and the 43/37% ($^{w}/_{w}$) interphase (Band 2) was washed in buffer and assayed for protein (section 2.2.7.). Each determination is the mean of three separate experiments.

seen at the 1.20/1.18 g ml^{-1} (Band 2) sucrose interphase if (i) the concentration of PYY cells seeded initially was sufficiently high (30 x 10^6 cells being the minimum); (ii) the cells were in good condition with normal growth and division, and finally (iii) if the cells were left to incubate for a minimum period of 40 h before harvesting.

3.3.5. The Preparation of Endoplasmic Reticulum (ER)

Endoplasmic reticulum prepared (section 2.2.6.) from [^{3}H]-glucosamine labelled BHK C13 and PYY cells was applied to a sucrose step-gradient identical to those used for the isolation of nuclear membrane and harvested following the same conditions of ultracentrifugation (2.2.5.1.). The [^{3}H]-glucosamine profiles from such gradients containing endoplasmic reticulum from PYY and C13 cells are shown in Fig. 19. Much of the labelled material originally applied to the gradient was found to have pelletted through the sucrose barriers. This accounts for 22.0% of the labelled PYY ER and 36.0% of the C13. A high proportion of the endoplasmic reticulum in each case also remains at the top of the sucrose gradient - 39.0% of PYY ER and 28.0% C13 ER. Small proportions were also found to band at the Band 1 (6.0% of PYY ER and 6.0% C13) and Band 2 (9.0% PYY ER and 8.0% of C13) nuclear membrane positions.

3.3.6. BHK Nuclear Membrane Prepared from Tween-washed Nuclei

[^{3}H]-glucosamine labelled C13 nuclei were washed in 1% $^v/_v$ Tween 80 detergent solution prior to nuclear membrane preparation (section 2.2.5.2.). The distribution of the label in the three nuclear membrane bands is shown in Fig. 20.

It is interesting that there is a shift in the [^{3}H]-glucosamine distribution on the sucrose gradient after the nuclei have been washed in Tween 80. Almost half of the labelled membrane preparation applied to the sucrose step-gradient for purification banded at the high

FIGURE 19 Sucrose Density Gradient Analysis of Endoplasmic Reticulum

Endoplasmic reticulum prepared (2.2.6.) from $[^3H]$-glucosamine-labelled BHK C13 and PYY cells were applied to a 50, 43 and 37% ($^w/_w$) sucrose step-gradient and centrifuged as for nuclear membrane isolation (2.2.5.1.).

Fig. 19 shows the gradient elution profile when fractions (0.5 ml) were collected and monitored for the distribution of the tritiated label by liquid scintillation analysis (2.2.3.1.).

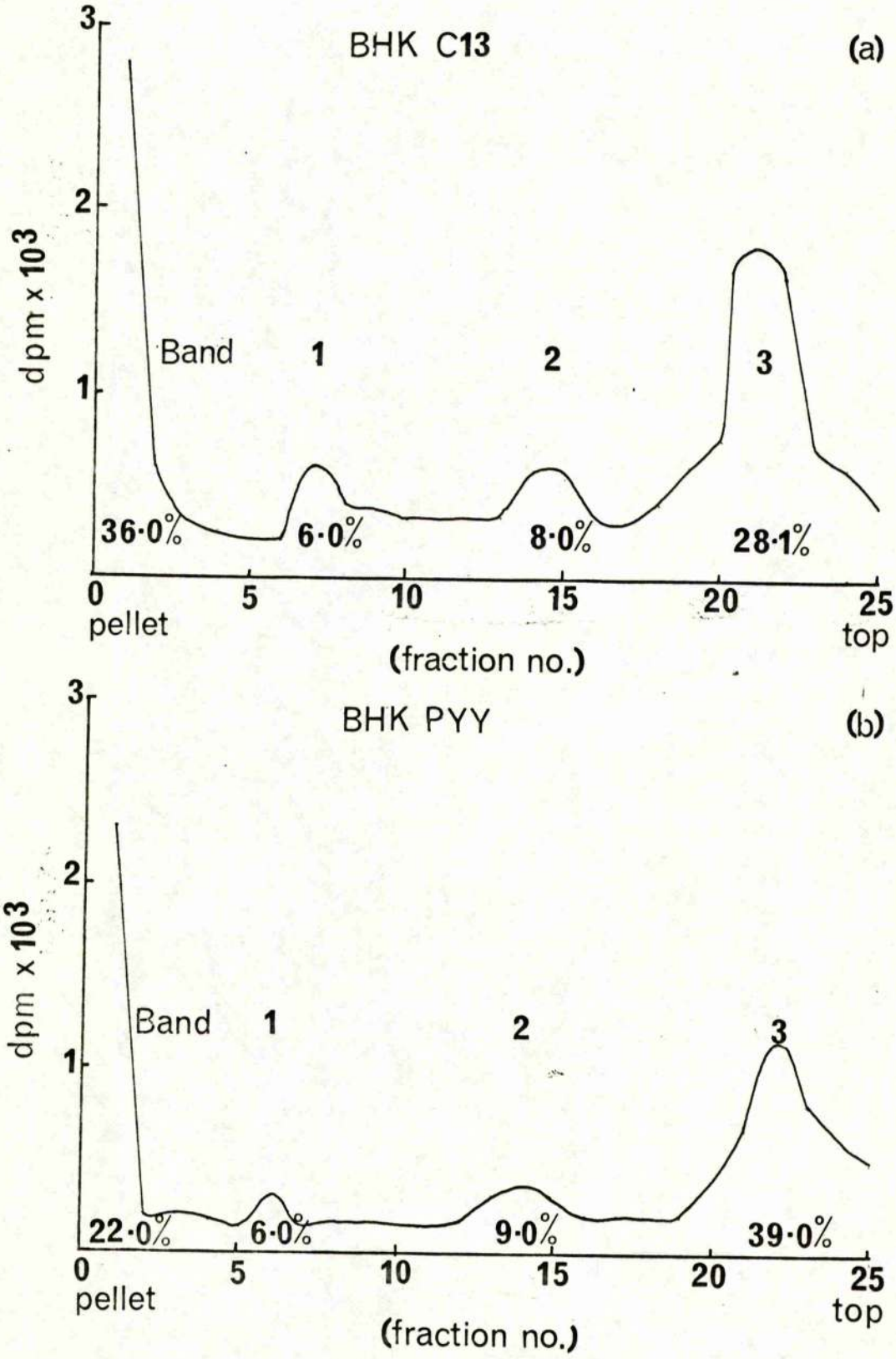
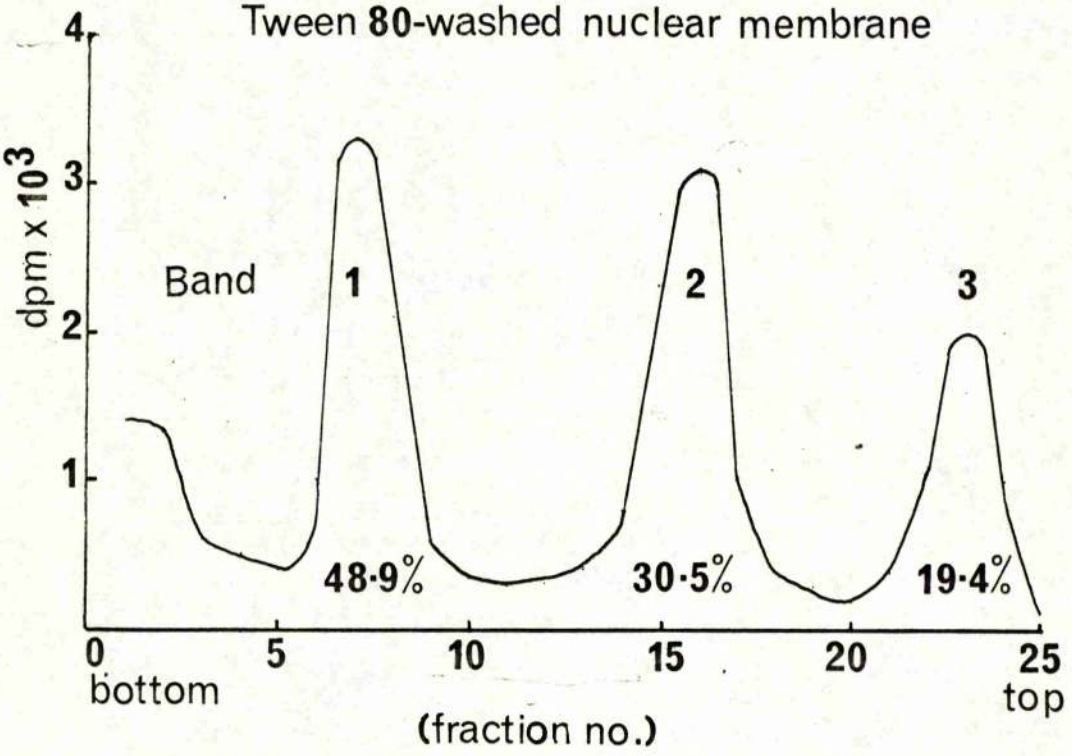

BHK C13
(a)
dpm x 10³
3
2
1
Band
1
2
3
36·0%
6·0%
8·0%
28·1%
0
5
10
15
20
25
pellet
top
(fraction no.)
BHK PYY
(b)
dpm x 10³
3
2
1
Band
1
2
3
22·0%
6·0%
9·0%
39·0%
0
5
10
15
20
25
pellet
top
(fraction no.)

FIGURE 20 Gradient Elution Profile of Nuclear Membrane Prepared from Tween 80 Washed C13 Nuclei

C13 cells (30×10^6) were grown in glucose-free EFC 10 and 50 mM in pyruvate and labelled with $[^3H]$-glucosamine (250 μCi) for 16 h. The nuclear pellet (2.2.4.2.) was incubated in 1% Tween 80 for 30 min prior to nuclear membrane isolation (2.2.5.2.). Fractions (0.5 ml) were pumped from the sucrose step-gradient and monitored for their radioactive content. The resulting profile is shown.


Tween 80-washed nuclear membrane
dpm x 10^3
4
3
2
1
Band
1
2
3
48·9%
30·5%
19·4%
0
5
10
15
20
25
bottom
top
(fraction no.)

density sucrose interface (Band 1, 48.9%). The rest of the [^{3}H]-glucosamine was divided thus: Band 2 (30.5%) and Band 3 (19.4%).

3.3.7. BHK C13 Nuclear Membrane Prepared from Citric-Acid Washed Nuclei

BHK C13 nuclei labelled with [^{3}H]-glucosamine were washed in 2.5% ($^w/_v$) citric acid prior to nuclear membrane preparation by the heparin method. The resulting distribution of the isotope throughout the final sucrose gradient is shown in Fig. 21.

The material found at Band 1 position constitutes 38.0% of the tritiated membranous material applied to the step gradient for purification. Of the remainder, 19.0% bands at position 2 and 16.0% at Band position 3.

Comparing the [^{3}H]-glucosamine distribution in each band in Fig. 21 with that membrane prepared by the same method without involvement of citric acid (Fig. 13(b)), it was found that a considerable amount of [^{3}H]-glucosamine labelled material has been removed from Bands 2 and 3. The total reduction of tritiated label from both bands was 25.0% relative to a control experiment such as that illustrated in Fig. 13(b).

It will be recalled that washing whole BHK C13 nuclei with 2.5% ($^w/_v$) citric acid removed 20.0% of the glycoprotein and phospholipid into the supernatant fluid after low speed centrifugation (Table 11).

3.3.8. Preliminary Characterisation of BHK C13 and PYY Nuclear Membrane Bands using Lens culinaris (Lentil Lectin)

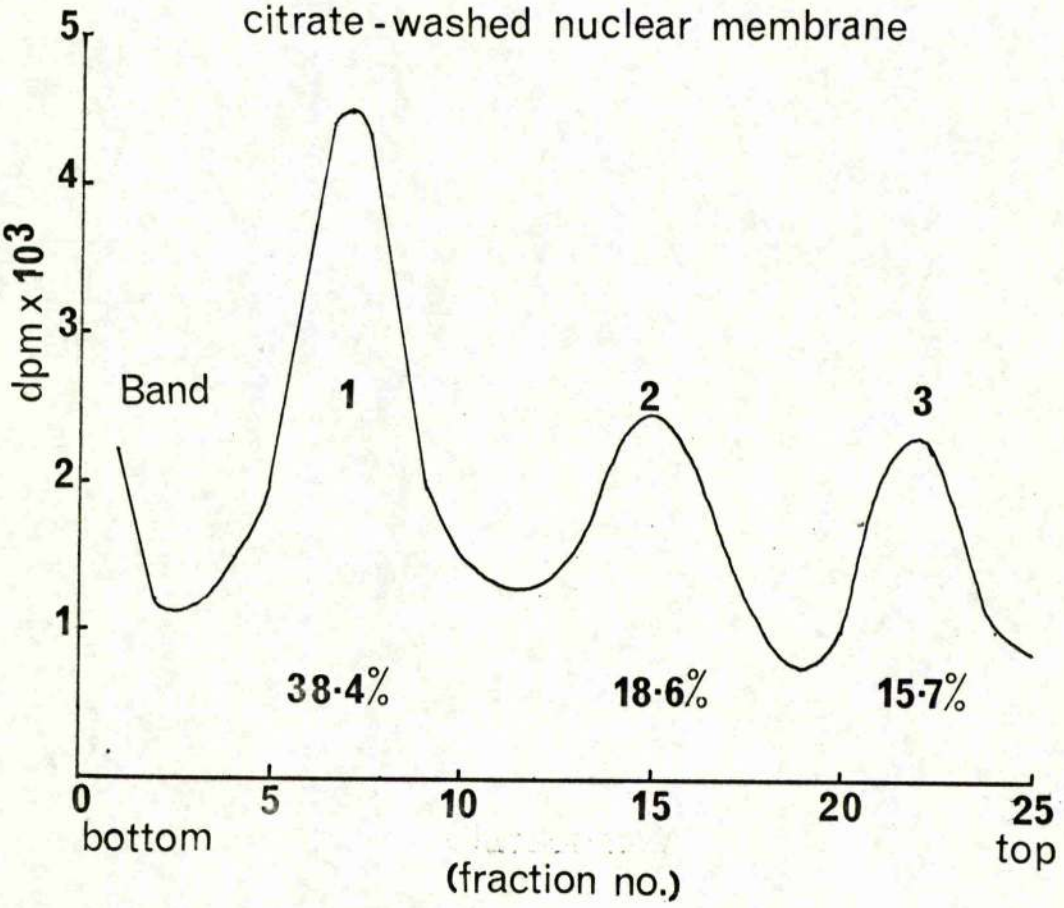
All six [^{3}H]-glucosamine labelled nuclear membrane bands (1,2 and 3) from C13 and PYY cells were loaded individually onto a column of lentil lectin (ex Lens culinaris) conjugated to Sepharose 4B (section 2.2.10).

The recovery of the radioactive label for each of the C13 nuclear

FIGURE 21 The Preparation of Nuclear Membrane from Citric Acid Treated BHK C13 Nuclei

BHK C13 cells (5×10^6) were grown in glucose-free EFC 10, 50 mM in pyruvate (50 ml) and labelled with $[^3H]$-glucosamine (250 μCi) for 16 h. The nuclei were resuspended in 2.5% $^w/_v$ citric acid (2 ml), incubated for 10 min on ice and centrifuged at 1000xg (av.) for 10 min. Nuclear membrane was prepared from the resulting nuclear pellet by the heparin method.

The profile of $[^3H]$-glucosamine collected in fractions (0.5 ml) from the sucrose density gradient is shown.


citrate-washed nuclear membrane
dpm x 10³
5
4
3
2
1
Band
1
2
3
38·4%
18·6%
15·7%
0
5
10
15
20
25
bottom
top
(fraction no.)

membrane bands was of the order of 90.0% of the material applied to the affinity column (88.0, 89.0 and 86.0% for Bands 1, 2 and 3 respectively). Approximately half as much material bound to the column as was unbound and eluted in the initial fractions (Fig. 22).

The PYY nuclear membrane bands proved in several repeats of this experiment to be less predictable. $[^3H]$-glucosamine labelled material applied to the column from PYY bands 1 and 3 was recovered to the extent of 84.0% and 97.0% respectively. In the case of Band 2, however, only 18.0% of the material was recovered, and all of this represented unbound species, in that the competing saccharide, α-methylglucoside, caused no further elution of $[^3H]$-label. Thus it was concluded that the $[^3H]$-glucosamine labelled material from PYY nuclear membrane Band 2 appeared to have a high affinity for the lentil lectin-Sepharose column and remains tightly bound to it.

3.3.9. Detection of Sialic Acid in C13 Membrane Bands

The procedure for the detection of sialic acid in nuclear membrane Bands 1 and 2 from C13 cells is described in section 2.2.9. 38.0% of the original material was recovered from the Dowex ion exchange column. The radioactivity profiles from the chromatograms carrying the $[^3H]$-glucosamine labelled bands are shown in Fig. 23 along with a diagrammatic representation of the chromatogram which bore the standard (non-labelled) sialic acid.

The isotope was detected in the same position on the chromatogram as was the unlabelled sialic acid. The recovery from the chromatogram of $[^3H]$-labelled species for Band 1 and Band 2 was 27.0 and 68.4% respectively; of this 22.6 and 63.5% of the total respectively could be accounted for at the position of sialic acid on the standard chromatogram. It would thus appear that Band 2 has a higher specific sialic acid content than has Band 1.

FIGURE 22 Preliminary Characterisation of BHK C13 and PYY Nuclear Membrane Bands using Lens culinaris (Lentil lectin)

A Burler (30×10^6 cells) each of C13 and PYY cells were grown in glucose-free EFC 10, 50 mM in pyruvate and labelled for 16 h with [^{3}H]-glucosamine (1 mCi). The three nuclear membrane bands from each cell line were isolated (section 2.2.5.2.). Each band was solubilised in 1% $^{w}/_{v}$ deoxycholate (0.5 ml) and applied to a column of Sepharose 4B coupled to Lens culinaris (2.2.10.).

Fractions (1 ml) were collected from the column, from which an aliquot (100 µl) was monitored for radioactivity (2.2.3.1.).

An example of the resulting profile is shown opposite.

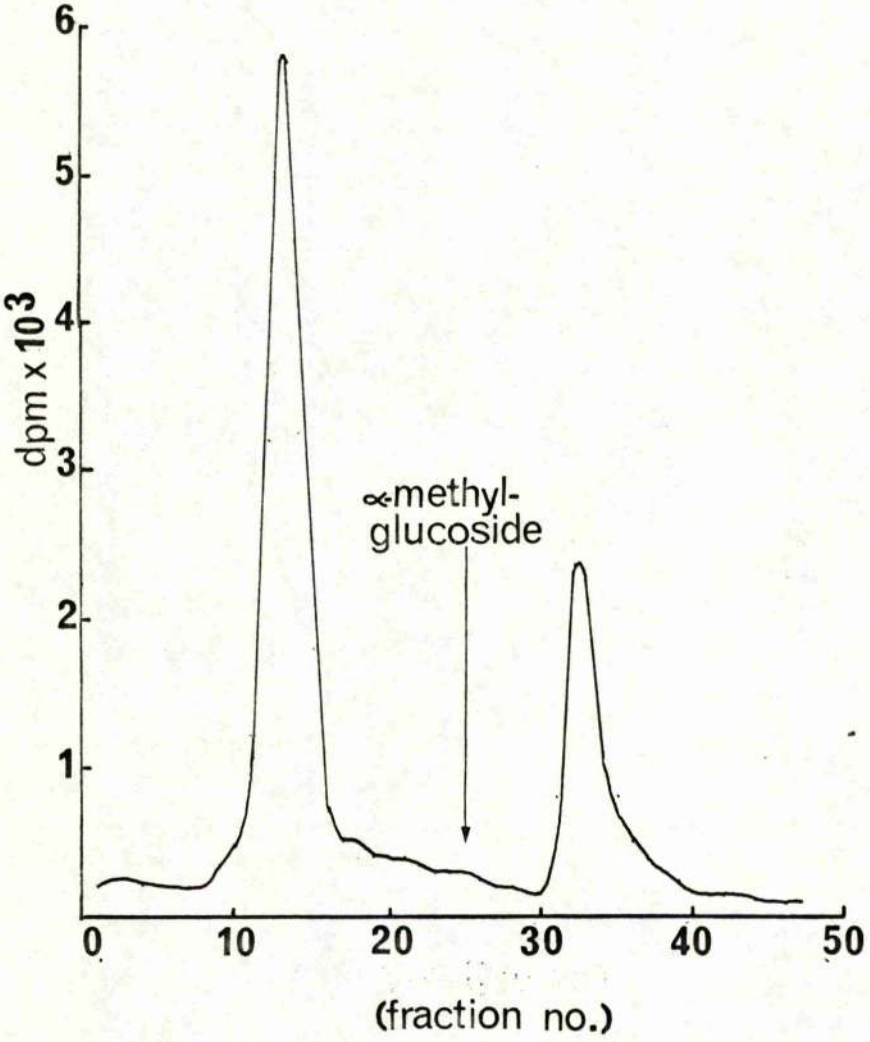
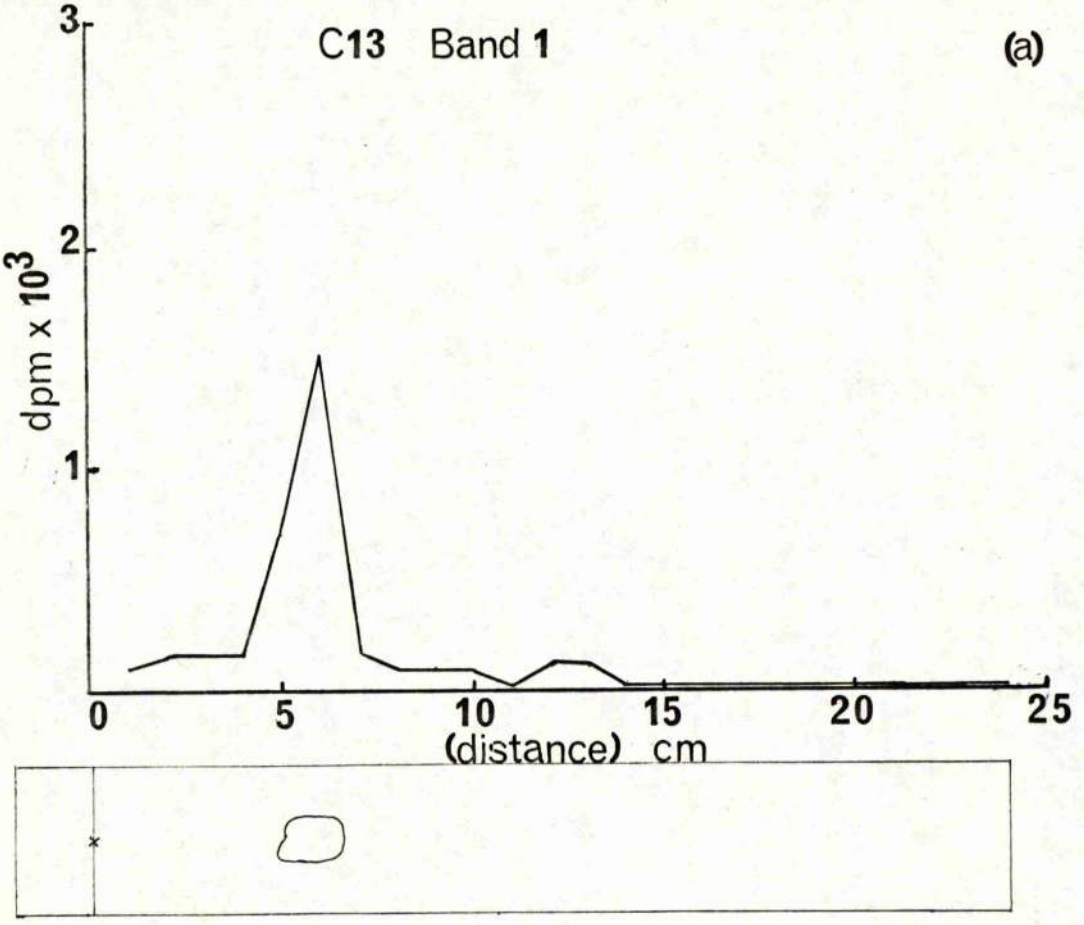
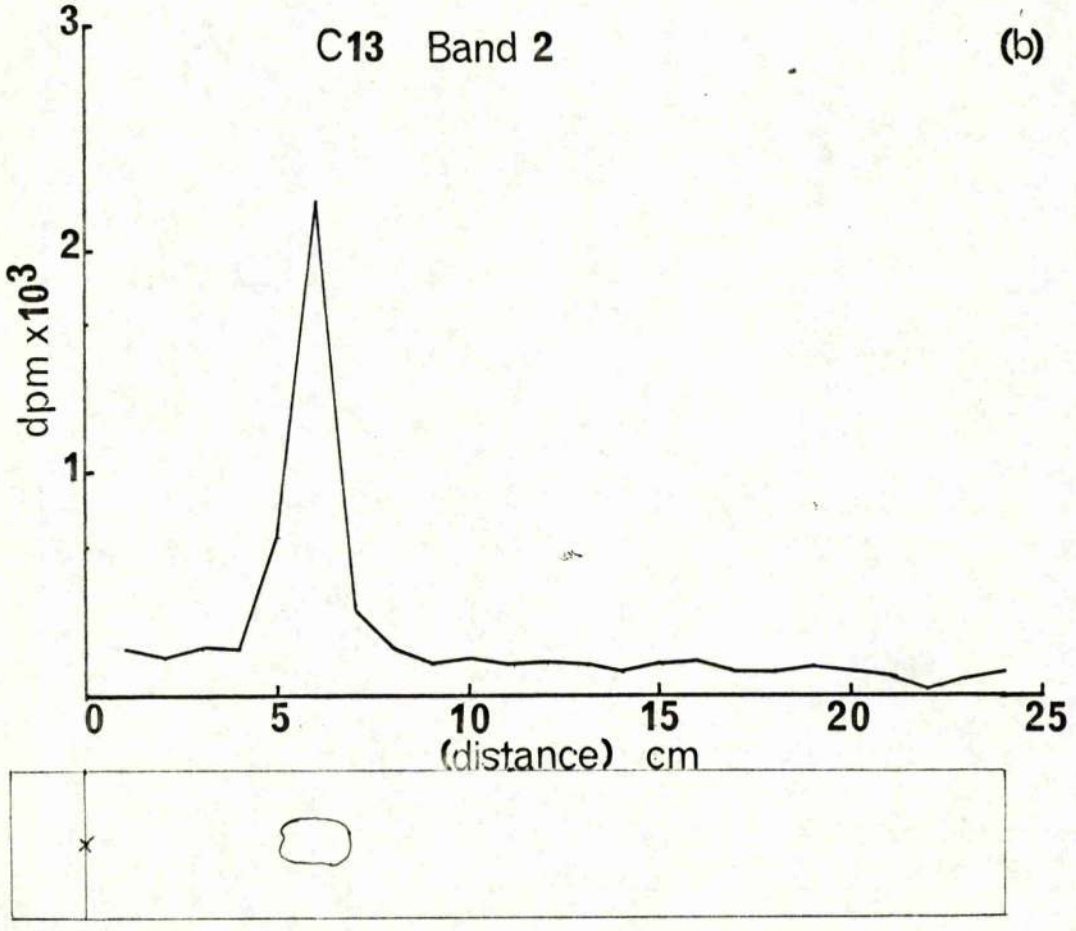

6
5
4
3
2
1
dpm x 10^3
∝-methyl-
glucoside
0
10
20
30
40
50
(fraction no.)

FIGURE 23 Detection of Sialic Acid on BHK C13 Nuclear Membrane

BHK C13 cells (30×10^6) grown in glucose-free EFC 10, 50 mM in pyruvate were labelled with $[^3H]$-glucosamine (1 mCi) for 16 h. Nuclear membrane Bands 1 and 2 were isolated (section 2.2.5.1.) and the sialic acid released by hydrolysis, separated on a Dowex ion exchange column and detected chromatographically as described in section 2.2.9.

The radioactively labelled chromatogram for each band was sliced into 1 cm strips and counted (2.2.3.2.).


C13 Band 1
(a)
dpm x 10^3
3
2
1
0
5
10
15
20
25
(distance) cm


C13 Band 2
(b)
dpm x10^3
3
2
1
0
5
10
15
20
25
(distance) cm

3.3.10. Amino Sugar Analysis of BHK C13 and PYY Nuclear Membrane Bands

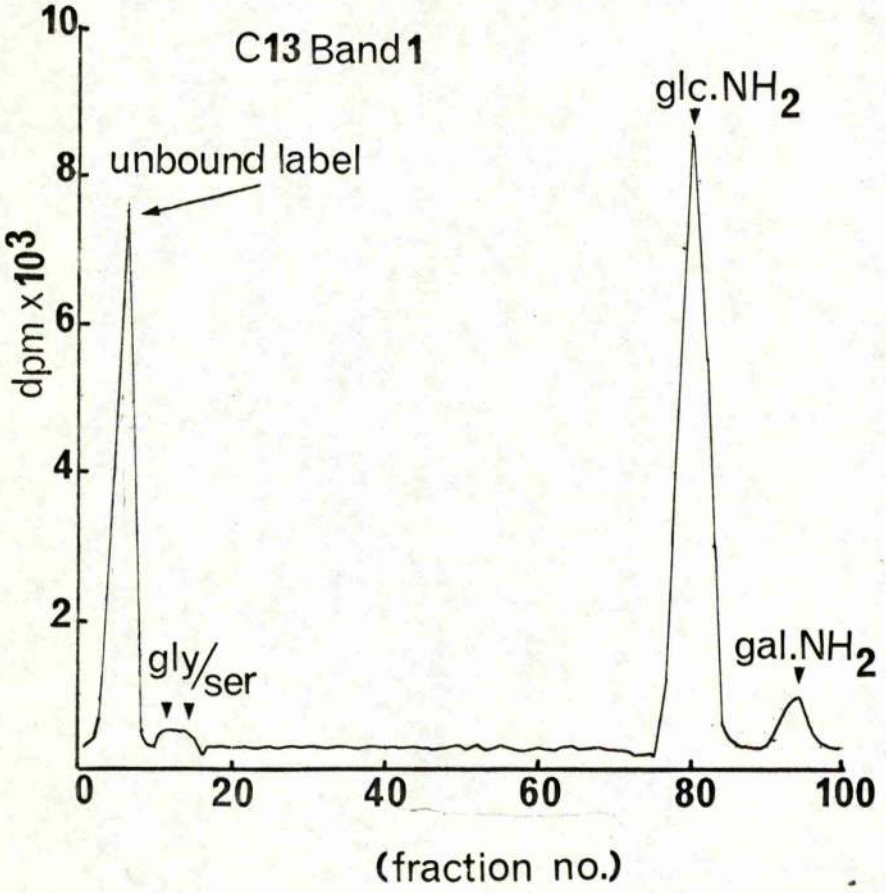
Amino sugar analysis was carried out (section 2.2.8.) on each (Bands 1, 2 and 3) $[^3H]$-glucosamine labelled bands of C13 and PYY nuclear membrane. Fractions (1 ml) were collected from the analyser and monitored for the presence of the isotope.

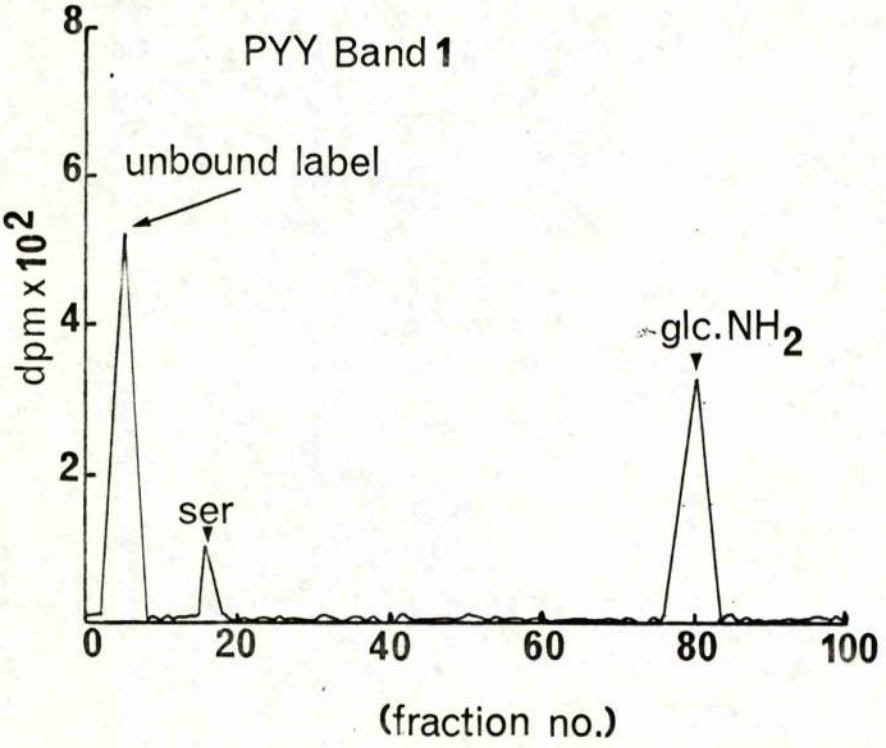
The elution profile of $[^3H]$-label in amino sugars detected in Band 1 of BHK C13 and PYY derived nuclei is shown in Fig. 24. Peaks of radioactivity were found coincidentally in the C13 Bands at the normal elution points of glucosamine and galactosamine. Galactosamine, however, was not detected in PYY nuclear membrane bands.

Small amounts of $[^3H]$-labelled glycine and serine could be detected on amino acid analysis of all bands from both C13 and PYY nuclei.

FIGURE 24 Amino Sugar Analysis of BHK C13 and PYY Nuclear Membrane

BHK C13 and PYY cells were grown and labelled as described for Fig. 23. Nuclear membrane was prepared as previously described (section 2.2.5.1.). Amino sugar analysis was carried out on each membrane band as described in section 2.2.8. Fractions (1 ml) were collected from the analyses and counted (2.2.3.1.). The $[^3H]$-glucosamine profiles for Band 1 of C13 and PYY cells are shown opposite.


C13 Band 1
glc.NH2
unbound label
dpm x 10^3
gly/ser
gal.NH2
10
8
6
4
2
0
20
40
60
80
100
(fraction no.)


PYY Band 1
unbound label
dpm x 10^2
glc.NH2
ser
8
6
4
2
0
20
40
60
80
100
(fraction no.)

DISCUSSION

4. DISCUSSION

4.1. The Metabolic Inhibitors, Hydroxyurea and Cytosine Arabinoside

Inhibitors have been considered useful as potential means of elucidating the mechanism of glycoprotein synthesis and extracellular transport (section 1.2.4.). Several drugs were utilised in this study to investigate the possibility of blocking labelled glucosamine uptake into the acid precipitable fraction of BHK cells, while allowing cell division and protein synthesis to occur. Two such drugs, hydroxyurea (Young et al., 1967(a) and 1967(b)) and cytosine arabinoside (Rama Reddy et al., 1971) are known inhibitors of DNA replication, as well as $[^3H]$-glucosamine uptake (Hawtrey et al., 1973 and 1974).

In order to ensure that glycoprotein biosynthesis was being studied in the normal situation of growing cells, it was preferable that inhibition of $[^3H]$-glucosamine incorporation into the acid precipitable material was established in the growing system, without invoking cessation of cell growth and division i.e. over a short incubation period and at a suitable concentration of the drug (Fig. 3). It would also be desirable that the inhibitor should have a limited inhibitory influence on protein synthesis whilst effectively blocking glycosylation of polypeptide chains.

In order to find a suitable concentration of $[^3H]$-glucosamine for labelling BHK cells, a pilot experiment was carried out in order to establish an approximate order of magnitude of the intracellular glucosamine pools. Fig. 3 illustrates that increasing concentrations of unlabelled glucosamine in the growth medium had no effect on i) the labelled intracellular acid soluble glucosamine pool or ii) the tritiated extracellular material. Only at high concentrations (10^{-4}M) of unlabelled glucosamine was there any inhibition of the intracellular

acid precipitable [^{3}H]-glucosamine label. This suggests that BHK C13 cells have themselves large endogenous pools of glucosamine.

Pulse labelling of BHK C13 cells with [^{3}H]-glucosamine and [^{3}H]-leucine in the presence of hydroxyurea indicated (Fig. 4) that although tritiated leucine incorporation was not significantly affected, [^{3}H]-glucosamine uptake was inhibited up to 40% between 2-7 h.

Lower concentrations of hydroxyurea than those used in this work (10 mM) have been reported to block DNA replication. Young et al. (1967(b)) found that hydroxyurea at concentrations as low as 1.32 mM inhibited incorporation of labelled thymidine into DNA by 84% over a similar period (8 h) to that illustrated in Fig. 4. This inhibition of DNA synthesis was also found (Young and Hodas, 1964) in mammalian and bacterial cells, but it did not alter the rates of synthesis of RNA or protein. Young et al. (1967(b)) also observed this effect in the sand dollar embryo over a 13 h incubation with hydroxyurea (1.32 mM).

The concentration of hydroxyurea chosen in these studies was that cited by Hawtrey et al. (1974). Under these conditions (10 mM hydroxyurea) they found an inhibition of incorporation of [^{3}H]-glucosamine by hamster embryo fibroblasts of 70% over 24 h.

A long term incubation (16 h) of BHK C13 cells with the drug at this concentration brought about an 87% inhibition of [^{3}H]-glucosamine and a 91% inhibition of [^{3}H]-leucine incorporation (Table 1). This contrasts with the findings of Hawtrey et al. (1974) who noted that simultaneously with the inhibition of [^{3}H]-glucosamine uptake (70%), protein synthesis was inhibited by only 12% over the same period.

Hawtrey et al. (1974) in a similar experiment using cytosine arabinoside (1 mM) found that incorporation of [^{3}H]-glucosamine into the acid precipitable cellular material was inhibited by 86%. Protein synthesis remained affected by 12%.

To investigate the effect of cytosine arabinoside (1 mM) on [^{3}H]-glucosamine incorporation into BHK cells in the short term a pulse label experiment (Fig. 5), similar to that carried out with hydroxyurea (Fig. 4), was undertaken. Cytosine arabinoside, under the conditions specified for Fig. 5 only began to inhibit incorporation of [^{3}H]-glucosamine into BHK Cl3 cells after 5 h. This is quite different from the results of Hawtrey et al. (1973). In their studies cytosine arabinoside (1 mM) inhibited incorporation of [^{3}H]-glucosamine into glycoproteins and glycolipids of hamster embryo fibroblasts by 85% after 3 h. The differing observations of this study and those of Hawtrey et al. (1973) must reflect the metabolic differences between the cell types used in each case.

By subjecting the cells to long periods (16 h) of incubation with hydroxyurea [^{3}H]-glucosamine uptake was inhibited; concurrently both DNA replication and protein synthesis were impaired and the cells no longer reflected the effect of the drug on glycoprotein synthesis alone. It is reasonable to suppose that since synthesis of the protein backbone has ceased, glycoprotein synthesis would not take place.

In the short term, since protein synthesis was unaffected by hydroxyurea and the inhibition of [^{3}H]-glucosamine into intracellular protein was inhibited by 33-40%, it would appear that the initial attachment of the saccharide residues to the peptide could be the point in the biosynthesis of glycopeptides at which hydroxyurea has its inhibitory effect.

It was decided on the basis of the information in Figs. 4, 5 and Table 1; and in the light of results obtained using 2-deoxyglucose (discussed below) not to pursue the use of hydroxyurea or cytosine arabinoside as a means of investigating [^{3}H]-glucosamine incorporation or the extracellular transport of the label.

4.2. The Inhibitor, 2-Deoxy-D-Glucose

The compound, 2-deoxyglucose has been used in recent years (Farkas et al., 1970; Ghandi et al., 1972; Melchers, 1973; Myers and Sartorelli, 1975) as an inhibitor of intracellular glucosamine uptake and secretion of glycoproteins. Although the precise mechanism of this inhibition is not known, 2-deoxyglucose appears to block the biosynthesis of the carbohydrate moieties of glycoproteins.

Melchers (1973) used 2-deoxyglucose to test the role of the biosynthesis of the carbohydrate groups in the synthesis, transport and secretion of IgG from tumour plasma cells. Thus the final concentration of 2-deoxyglucose in the growth medium of BHK C13 cells was chosen as 20 mM, in keeping with the concentration used by Melchers (1973) and other workers (Ghandi et al., 1972).

Fig. 6 shows that a 1 h incubation of cells with 2-deoxyglucose (20 mM) prior to isotopic labelling inhibits incorporation of $[^3H]$-glucosamine into the intracellular acid precipitable material by 80%. Uptake of $[^3H]$-leucine, which was simultaneously monitored under the same conditions, was inhibited by a corresponding 45%. This initial result suggested that 2-deoxyglucose would prove a more useful tool in this investigation than either hydroxyurea or cytosine arabinoside, in that it efficiently blocked glycoprotein synthesis by inhibition of $[^3H]$-glucosamine incorporation, while allowing protein synthesis to occur, albeit less efficiently.

The BHK C13 cells were grown in glucose-free Eagle's medium supplemented with pyruvate (50 mM), as glucose has been shown to compete with 2-deoxyglucose (Kaluza et al., 1973). Kaluza et al. (1973) found 2-deoxyglucose inhibited virus multiplication less effectively in cells incubated in medium containing glucose or mannose than in medium containing no or other sugars. They demonstrated that the up-

take and incorporation of [^{3}H]-2-deoxyglucose into virus infected cells was influenced most severely by glucose, somewhat less by mannose and not at all by fructose or pyruvate.

It was found (Table 2) that increasing the time of exposure of cells grown on glucose-free EFC 10 to 2-deoxyglucose (20 mM), increased the degree of inhibition of [^{3}H]-leucine incorporation into the cells. The inhibition ranged from 43% after 24 h to 60% after 7 h in the presence of the inhibitor. At this 2-deoxyglucose concentration the inhibition of [^{3}H]-glucosamine incorporation remained consistently high at 86-90% throughout the experiment.

Comparing these results with those obtained from a similar experiment in which the BHK C13 cells were grown in normal EFC 10 (i.e. glucose present) (Table 3), the inhibition of incorporation of [^{3}H]-glucosamine by 2-deoxyglucose (20 mM) was reduced to 58% (after 1 h) - 70% (after 7 h). 2-Deoxyglucose is an antimetabolite of glucose (Kaluza et al., 1973) and the [^{3}H]-labelled compound was incorporated into viral proteins of Semliki Forest virus infected chick fibroblasts. This finding conflicts with the suggestion made by Kipris and Cori (1959) that 2-deoxyglucose is not significantly metabolised beyond the level of the 6-phosphate derivative in animal cells.

The effect of 2-deoxyglucose over a longer period of time (17 h) on [^{3}H]-glucosamine and [^{3}H]-leucine incorporation into the intracellular acid precipitable pool is shown in Table 5. The prolonged incubation made little difference to the inhibition of both [^{3}H]-glucosamine uptake, which remained high (82%), and [^{3}H]-leucine (63%).

The influence of 2-deoxyglucose on [^{3}H]-glucosamine incorporation into the intracellular acid soluble pool and the secretion into the extracellular growth medium was also investigated (Table 4). In this experiment the high inhibition of incorporation of radioactivity

into the acid precipitable fraction was confirmed, whilst the inhibition of incorporation into the acid soluble pool was found to be less (60%). 2-Deoxyglucose did not appear to influence the acid precipitable labelled material secreted into the extracellular medium. It is possible that 2-deoxyglucose was inhibiting uptake by acting as a competitor of glucosamine, as was found for fructose and maltose by Kuo and Lampen (1972) (cf. Kaluza et al., 1973).

Similar experiments to those carried out with [^{3}H]-glucosamine were undertaken with [^{3}H]-fucose, since this is a sugar residue found specifically in glycoproteins and glycolipids. It was considered that fucose incorporation would give some insight into the nature of the effects of 2-deoxyglucose inhibition of intracellular attachment of the tritiated sugar into glycoprotein.

A pulse label experiment was carried out using [^{3}H]-leucine (Table 6(a)) and [^{3}H]-fucose (Table 6(b)). The inhibition of incorporation of [^{3}H]-leucine into the acid precipitable cellular fraction was 43% after a 1 h incubation with 2-deoxyglucose (20 mM), rising to 61% after 7 h. [^{3}H]-fucose incorporation was so low after 1 h of labelling that the presence of the inhibitor had a less marked effect on the intracellular radioactivity. In spite of this, Table 6(b) shows an inhibition of 35% after 7 h with 2-deoxyglucose in the medium. This contrasts with the uptake of [^{3}H]-glucosamine, which after 7 h incorporation was reduced by 90% in the presence of 2-deoxyglucose.

Fucose is characteristically found at the terminal position of oligosaccharide chains. Sugar residues such as fucose and the sialic acids are incorporated into glycoproteins at a later stage of synthesis than the initiating and core sugars, such as glucosamine. It would therefore appear that fucose was being incorporated into the pre-formed glycoprotein pools just prior to their export. Hence, in the case of

Table 7, after 1.5 h labelling (inhibitor present for 7.5 h), the pre-formed glycoprotein pool was still quite large, as there was only a 40% inhibition of $[^3H]$-fucose incorporation, compared with 90% $[^3H]$-glucosamine incorporation under the same conditions after 7 h. $[^3H]$-Fucose labelling for 2.5 h (inhibitor present for 8.5 h) showed an increased inhibition (60%) of label incorporation. At this time the pre-formed glycoprotein pool would have been diminished further by export and thus $[^3H]$-fucose incorporation was inhibited further.

It would appear that there may be several possible mechanisms of 2-deoxyglucose action, one being the inhibition of the initial attachment of glucosamine to the protein backbone, thereby inhibiting further glycoprotein synthesis. An alternative explanation may be that 2-deoxyglucose inhibits the migration of the polypeptide chains from the membrane-bound polyribosomes into the cisternae of the rough endoplasmic reticulum, as found by Melchers (1973) in tumour plasma cells. In the case of fucose, 2-deoxyglucose only prevents incorporation indirectly by depleting the intracellular glycoprotein pools.

The inhibition of uptake of $[^3H]$-fucose by 2-deoxyglucose into the acid soluble pool was 26% (Table 7) after 7.5 h of inhibitor presence, compared with 60% for $[^3H]$-glucosamine after 4 h (Table 4). The transport of $[^3H]$-glucosamine into the cell appeared to have been inhibited to a greater extent than in the case of $[^3H]$-fucose. In common with Kuo and Lampen (1972), it was found that 2-deoxyglucose inhibited the uptake of saccharide residues by the cells.

The double label experiment depicted in Table 8 effectively confirmed the foregoing effects of 2-deoxyglucose on both $[^3H]$-glucosamine (Table 4) and $[^3H]$-fucose (Table 7) uptake and metabolism. 2-Deoxyglucose did not affect pre-labelled (tritiated in this case) glucosamine acid soluble and acid precipitable pools, nor the subsequent extra-

cellular secretion of acid-precipitable material (plates 1 and 2, Table 8).

In common with the [^{3}H]-glucosamine incorporation experiments shown in Table 4, 2-deoxyglucose inhibited the formation of intracellular acid precipitable and soluble pools (^{14}C-labelled) by approximate factors of 10 and 5 respectively, irrespective of whether there was pre-labelling (plates 5 and 6) or not (plates 3 and 4). In all experiments the transport of extracellular acid precipitable material was unaffected by 2-deoxyglucose over 2.5 h.

It will be seen from Tables 4 and 7 that the secretion of both [^{3}H]-glucosamine and [^{3}H]-fucose acid precipitable fractions was inhibited to a small degree by 2-deoxyglucose. In the case of [^{3}H]-fucose, a maximum of 23% inhibition was achieved only after a period of 8.5 h. For shorter time periods, inhibition of secretion of both [^{3}H]-glucosamine and [^{3}H]-fucose containing acid precipitable material was very much reduced (below 10%). The higher inhibition of 23% in the case of [^{3}H]-fucose was considered to be due to the previously discussed limitation on pre-formed glycoprotein pools caused by the inhibition by 2-deoxyglucose of intracellular glycoprotein biosynthesis.

The extracellular secretion of [^{3}H]-glycosylated material was not inhibited by 2-deoxyglucose. This finding is in agreement with the studies of Melchers (1973) on tumour plasma cells and their secretion of IgG.

It will be noted from Table 8 that while 2-deoxyglucose had no influence on the secretion of tritiated, glycosylated, acid precipitable products into the medium, there was a substantial level of radioactivity detected in this extracellular fraction. It was considered of interest to investigate the biochemical nature of this extracellular material to further elucidate the fate of the tritiated label, once incorporated

into the cellular system.

To this end the experiments (section 3.1.13.) were performed and the results obtained are summarised in Fig. 7.

High molecular weight $[^3H]$-glycosylated species were isolated from the medium by gel filtration (Fig. 7(a)) of BHK cells in the absence of 2-deoxyglucose. However, in the presence of 2-deoxyglucose these high molecular weight species were much reduced (Fig. 7(b)). This result was unexpected in the light of previous results shown in Tables 4, 7 and 8, in which 2-deoxyglucose did not influence the secretion of the glucosamine label. Fig. 7(b) shows that most of the tritiated material which continued to be secreted in the presence of 2-deoxyglucose was of lower molecular weight (though acid precipitable).

Investigation of the nature of the high molecular weight labelled extracellular material was subsequently carried out (Fig. 8) using the enzymes pronase and hyaluronidase. Pronase treatment led to the formation of low molecular weight peptides with no associated $[^3H]$-glucosamine (Fig. 8(b)). Subjection of the labelled extracellular material to hyaluronidase resulted in a marked cleavage of the high molecular weight fraction into lower molecular weight species which on the basis of absorption at 280 nm proved to be non-proteinaceous (Fig. 8(c)). There were, however, high molecular weight glycopeptides remaining which were not susceptible to cleavage by pronase.

Further investigation (post-pronase treatment) of the extracellular high molecular weight material by DEAE cellulose chromatography (Fig. 9) found peaks of radioactivity to be eluted by a linear gradient of ammonium acetate in the same positions as observed for hyaluronic acid and choindroitin sulphate (Stein et al., 1975; Kraemer, 1971).

The extracellular glycosylated secretion therefore could be attributed, on the basis of these experiments, to at least three

molecular types namely i) glycoproteins and/or glycopeptides, ii) hyaluronic acid and iii) choindroitin sulphate.

4.3. Nuclear Membrane Glycoproteins

A second aspect of this study was to investigate the existence of glycoproteins in the nuclear membrane of normal BHK Cl3 cells, and if so present to see if they reflect the same differences brought about by transformation as has been demonstrated in plasma membrane glycosubstances (section 1.3.2.).

In the first instance it was important to isolate BHK cell nuclei which were intact, free (i.e. non-aggregating) and devoid of intracellular contamination. The non-ionic detergent Nonidet P40 was used in the preparation of nuclei (section 2.2.4.1.) at the outset of this work. However, since there had been reports (Sadowski and Howden, 1968; Keshgegian and Glick, 1973) of Triton X-100 (a non-ionic detergent similar in chemical structure to Nonidet P40) removing, not only cytoplasmic contamination, but the entire outer nuclear membrane. The solubilisation studies described in section 3.2. confirm these results (Table 10). Further $[^3H]$-ethanolamine labelled lipids (89.0%) were removed by the non-ionic detergent Triton X-100. This high solubilisation of labelled lipids indicated the total breakdown of the nuclear membrane under the influence of Triton X-100.

It was necessary, therefore, to prepare nuclei by an alternative method which did not involve the use of detergents (section 2.2.4.2.). Comparison by SDS polyacrylamide gel electrophoresis of whole nuclei prepared by both methods illustrated (Fig. 10) that major glycoprotein and glycolipid components were removed as a result of subjecting the nuclei to detergent treatment as was demonstrated in the solubility studies (Tables 9 and 10). Gels stained with coomassie blue (Fig. 10(a) and (b)), illustrated that some high molecular weight components were seen to be absent from the Nonidet P40 (Fig. 10(b)) treated nuclei. However, since the major protein bands appear to be similarly

situated in both preparations, it would seem that those proteins and glycoproteins removed by the detergent were minor components of the nucleus. Fig. 11 confirms that the [^{3}H]-glucosamine label was being incorporated into nuclear glycoproteins and glycolipids, as these gels have the same radioactivity profile as SDS gels labelled with [^{3}H]-fucose.

The findings in Fig. 10 support the work published by Aaronson and Blobel (1974) in that Triton X-100 solubilised both the nuclear phospholipid (over 95%) and the nuclear membrane protein (10%) of isolated rat liver nuclei (cf. Table 10 Triton X-100 removed 89.0% [^{3}H]-ethanolamine). Aaronson and Blobel (1974) also showed that the shape and ultrastructure of membrane denuded nuclei continued to be maintained. It would therefore not be obvious from the use of the light microscope that nuclei prepared using such detergents were devoid of their membrane.

The detergent method of nuclear preparation was abandoned and the tris-sucrose method was subsequently employed. Solubility studies (Tables 9 and 10) indicated that glycoproteins and glycolipids associated with the nuclear membrane might be selectively removed by non-ionic detergents. This has been found by other workers from studies on plasma membranes (Yu et al., 1973, Butters and Hughes, 1974).

The preliminary experiments carried out under the heading of "Solubility Studies" (section 3.2.) were undertaken with a view to isolating BHK nuclear membranes which were free from either cytoplasmic or, more likely, endoplasmic contamination.

Many methods are available for the isolation of the nuclear membrane from a variety of tissues (Franke, 1966; Kashnig and Kasper, 1969; Franke et al., 1970; Agutter, 1972; Monneron et al., 1972; Stavy et al., 1973) and the most favourable conditions of successful

preparation are dealt with in two recent books (Maddy, 1976; Birnie, 1976). The two methods routinely employed in this study were those of Bornens (1973) using heparin and a much modified version of the Kasper and Kashnig method (1969) involving sonication of the nuclei (section 2.2.5.). In each case the nuclear membrane was purified through a sucrose step-wise density gradient (Fig. 12). Most of the membrane material was seen to band at the 1.22/1.20 g ml^{-1} interface. A lesser proportion of material was found at the 1.18/1.20 g ml^{-1} interface. Crude nuclear membrane material remained at the top of the gradient (Fig. 12, Band 3) and a small pellet was found at the bottom. Bornens (1973) similarly reported that the bulk of nuclear membrane banded at the 1.22/1.20 density interphase. No mention was made of other bands elsewhere in the gradient. Kasper and Kashnig (1969) reported that the nuclear membrane formed a band at two interfaces 1.20/1.18 and 1.18/1.16 and further that the pellet consisted of intact nuclei and nucleoli.

On the basis that Bands 1 and 2 were at the densities of previously reported preparations of nuclear membrane, it was decided to accept this premise, arbitrarily, for the moment. The nature of Band 3 will be considered later in the discussion.

The relative merits of each preparation can be judged from the gradient elution profile of labelled nuclear membrane (Fig. 13), a higher recovery of $[^3H]$-glucosamine was found using the heparin method. It was noted that the material in Band 3 was less than the Band 3 remaining after preparation of the nuclear membrane by sonication. This may be a direct consequence of heparin action. Polyanions such as heparin cause morphological changes in the nuclei (e.g. swelling) and appear to release nuclear chromatin resulting in the complete release of the nuclear DNA (Kraemer and Coffey, 1970(a) and (b)).

Bornens (1973) reported that nuclear membrane prepared using heparin was completely free from nuclear contamination as judged from electron microscopy. It was found in the studies described in this thesis that after treatment of the nuclei with both DNAase and heparin a larger pellet of material (cytoplasmic and nuclear) was obtained after centrifugation through sucrose than when DNAase alone was used (sonication method).

All the nuclear membrane bands isolated by the heparin procedure were subjected to polyacrylamide gel electrophoresis and the coomassie blue stained gels are shown in Fig. 14. The protein patterns of Bands 1 and 2 from the C13 nuclear membrane preparation are very similar, as are those of Band 3, although some low molecular weight components appear to be less obviously present. Insufficient material was present on the gel of Band 2 of the PYY preparation for photography, but by observation the protein patterns of Bands 1 and 2 appeared to correlate closely. Very much less material was present in the pellet from the PYY nuclear membrane sucrose gradient than from that of C13. This, however, may be due to the fact that the amount of nuclear material generally from PYY cells was consistently less than that from C13 cells, as may be seen from the protein estimation of the two main nuclear membrane bands in Table 13.

PYY Band 3 always had the most material of the three PYY bands, which might account for less protein being released into the two lower bands. Comparison of all three membrane bands on the basis of protein staining revealed no major differences, either for C13 or for PYY nuclear membranes. It is possible that the differences in banding density could indicate separation of the double (nuclear) membrane structure or they could simply reflect differing degrees of contamination of the membrane. It was not possible to tell from the electron micrographs

obtained (Fig. 18), negative stained preparations however may have revealed the differences between the membrane bands.

Another notable feature was the similarity in protein characteristics between the normal BHK C13 nuclear membrane and that of the transformed counterpart PYY.

In order to see if these similarities applied to the nuclear membrane glycoprotein $[^3H]$-glucosamine labelled nuclear membrane bands were subjected to SDS polyacrylamide gel electrophoresis and the gel profiles compared (Figs. 15 and 16).

A comparison of Fig. 15 and 16 reveals that there are more glycosylated components in the C13 nuclear membrane bands than in PYY. Of the former, Band 1 shows six labelled components, while Bands 2 and 3 have seven and the pellet three $[^3H]$ -components which were common to all three bands. Bands 1 and 2 of the PYY nuclear membrane exhibit four $[^3H]$-glucosamine labelled species, while Band 3 and the pellet display five peaks of radioactivity.

Glycolipid is present in all the nuclear membrane bands, but proportionately reduced in the pellet. The molecular weight of all the glycosylated species are given in section 3.3.2.

Two high molecular weight tritiated components are common to nuclear membrane Bands 1, 2 and 3 of both cell types; and the three low molecular weight tritiated species common to all C13 nuclear membrane bands are not found in the PYY nuclear membranes.

Sieber-Blum and Burger (1977) identified nine proteins in the nuclear membranes of CHO cells on SDS-polyacrylamide gel electrophoresis which contained substantial amounts of amino sugars. Tritiated components of both the PYY and C13 cells have similar molecular weights to five of the nine nuclear membrane proteins found to be glycosylated in CHO nuclei by Sieber-Blum and Burger (1977).

These findings contrast with those of Jackson (1976) who was unable to detect glycopeptide on the chick erythrocyte nuclear membrane by periodate - Schiff staining. It is possible, however, that this technique was insufficiently sensitive to detect such minor components of the nuclear membrane.

The most likely contaminant of any nuclear preparation would be endoplasmic reticulum (ER), which is continuous with the nuclear membrane in the cell. A preparation of ER was therefore prepared and applied to a sucrose density stepwise gradient, similar to that used for the isolation of nuclear membrane bands (Fig. 19). Most of the labelled material applied to the gradient in the case of both C13 and PYY cells, either pelletted or remained in Band 3. Only very small amounts (6-9%) appeared in either Band 1 or Band 2 (section 3.3.5.).

It appeared from this result that Bands 1 and 2 were, in fact, nuclear membrane, while Band 3 and the pellet material contained most of the contaminating endoplasmic reticulum. It was not possible from the electron micrographs obtained to distinguish many characteristic features of nuclei except the aggregates of nuclear pore complexes. The presence of the double nuclear membrane structure could not be observed in micrographs although ribosomes were found in abundance in all bands. It is felt that negative staining would have provided micrographs of a better quality thus permitting easier morphological assessment of the membrane preparations.

It may be recalled from prior experiments with detergents and membrane perturbants (section 3.2.) that citric acid removed 20% of both labelled phospholipid (Table 11) glycoprotein material from intact nuclei and that the non-ionic detergent Tween-80 removed approximately 20% of labelled protein and glycoprotein (Table 12) from intact C13 nuclei.

Preparations of nuclear membrane from C13 nuclei treated with Tween-80 (Fig. 20) and citric acid (Fig. 21) were compared with membrane for untreated nuclei (Fig. 13(a)) to investigate the ability of Tween-80 and citric acid to remove ER contamination from the nuclear membrane.

Tween 80 appeared to release nuclear membrane trapped in Band 3, since a proportion of $[^3H]$-glucosamine counts disappeared from Band 3, while the radioactivity in Band 1 increased. Tritiated glucosamine counts in Band 2 did not appear to be affected by washing of C13 nuclei with Tween-80.

Citric acid, on the other hand, removes $[^3H]$-glucosamine label from both Bands 2 and 3 by a total of 25%. Since citric acid has been used to isolate the outer nuclear membrane (Smith et al., 1969) it may be that Band 2 was rich in the outer nuclear membrane.

When BHK C13 nuclear membrane was prepared (heparin) from citric acid treated nuclei, a slight shift in the $[^3H]$-glucosamine profile was observed (Fig. 21) relative to untreated nuclei (Fig. 13(b)). $[^3H]$-glucosamine label was removed by citric acid from Band 2, while the relative differential in radioactivity between Bands 1 and 3 was maintained. Thus Band 2 may have represented both the inner and outer nuclear membrane intact.

Limited characterisation of the nuclear membrane glycoprotein was performed and the results presented in section 3.3.8. - 3.3.10.

Detergent solubilised nuclear membrane bands of both BHK cell lines were subjected to affinity chromatography on Sepharose 4B - Lens culinaris haemagglutinin conjugate. This lectin is specific for the glycosides of D-glucose and D-mannose.

Fig. 22 shows a typical $[^3H]$-glucosamine elution profile of any of the labelled C13 nuclear membrane bands when loaded onto the column. Binding of labelled material to the column was reversed by competition

with 2-methylglucoside in the eluting buffer. Approximately half as much of the labelled material bound to the column as was eluted in the initial fractions. There was a high recovery of the $[^3H]$-glucosamine label from Cl3 nuclear membrane Bands 1, 2 and 3.

A similar result was obtained from the PYY nuclear membrane Bands 1 and 3. Band 2, however, proved to be different. A small percentage of the $[^3H]$-labelled material applied to the column could be accounted for in the eluted fractions, and that was exclusively in the unbound material. It is a possibility that this nuclear membrane band (PYY Band 2) had a high affinity for the lentil lectin - sepharose and remained tightly bound to it.

These preliminary findings showed, firstly, that they confirmed the results of previous experiments that glycoproteins exist on the nuclear membrane. Secondly, it was the first indication that PYY nuclear membrane Band 2 differs from Bands 1 and 3 of PYY nuclei, as well as those of Cl3 nuclei. Since Band 2 appeared to be (Fig. 21 and Table 11) largely outer nuclear membrane, then this result and those in Figs. 15 and 16 indicate some difference in the outer nuclear membrane of BHK Cl3 and their transformed PYY counterparts.

$[^3H]$-Glucosamine was used to label nuclear membrane glycoprotein components. Tritiated label was detected in the nuclear membrane in three forms - i) sialic acid, ii) glucosamine and iii) galactosamine.

Fig. 23 illustrates that BHK Cl3 nuclear membrane Band 2 appears to have a higher specific sialic acid content than Band 1. (The chromatography was not carried out on PYY nuclear membrane).

Amino sugar analysis of the nuclear membrane Bands 1 and 2 from both BHK cell lines was carried out (Fig. 24). Radioactivity was detected coincidentally at the normal elution points of glucosamine and galactosamine, to a lesser extent in both Cl3 Bands 1 and 2. The

The label was not detected in galactosamine in either of the PYY nuclear membrane Bands 1 and 2 although glucosamine was present in both bands. This may be a further difference between the cell types. It may, however, be due to the low degree of labelling of the membrane in the transformed cell type. Similarly, Kashnig and Kasper (1969) were only able to detect glucosamine in the nuclear membrane of rat liver cells.

While quantitation of such experiments would enhance the present sparce knowledge of the nuclear membrane, they, nevertheless, served to confirm that the $[^{3}H]$-glucosamine label used in this study was incorporated into the oligosaccharides of glycoproteins and glycolipids of the nuclear membrane.

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