EXAMINATION OF SOME PROTEIN CONTAINING FRACTIONS FROM HUMAN ERYTHROCYTE GHOSTS FOR GLUCOSE BINDING AS PART OF THE HEXOSE TRANSPORT SYSTEM

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ROY PERCY EADY

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University of London

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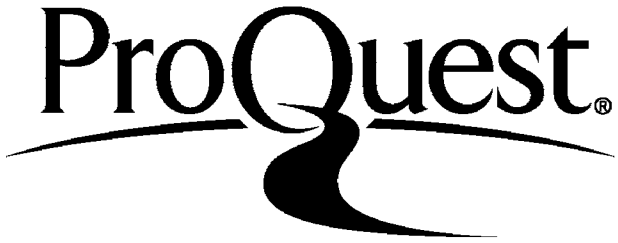

ABSTRACT

Kinetic studies on the penetration of human red cell membranes by hexoses have shown that transport occurs by a carrier mechanism. Little is known about the biochemical aspects of this carrier mechanism but the carrier is probably lipid, protein or a combination of the two. Studies were carried out to investigate the possibility of membrane proteins from red blood cells being involved in carrier function.

Lipid-free membrane proteins were obtained by n-butanol extraction of human erythrocyte stroma. Electrophoretic techniques showed that these proteins would associate with glucose. However, this was demonstrated by both D- and L-glucose.

Specific binding of D-glucose by a lipoprotein material situated at the oil : water interface during butanol extraction was observed. This binding, demonstrated by retention of radioactivity on a millipore filter, could be reduced by about 50% using high concentrations of competitive sugars.

The non-ionic detergent, Triton X-100, solubilized both the membrane proteins and lipids. Precipitation of the solubilized material with 50% saturated ammonium sulphate in the presence of radioactive glucose indicated that a component could bind D-glucose in preference to L-glucose. This component was probably protein or lipoprotein as precipitation with 10% trichloroacetic acid reduced the binding of D-glucose. Assuming all the D-glucose binding, (1.14×10^{-7} g per mg of protein), was involved in carrier function, each red cell would contain 95,000 active sites. Although bovine serum globulins, (and red cell membrane proteins solubilized by butanol extraction), also showed some preferential binding of D-glucose on precipitation by ammonium sulphate, the binding activity was much lower than the Triton X-100 extract. D-glucose binding to the Triton X-100 material could be reduced using the non-competitive inhibitor mercuric chloride and high concentrations of competitive sugars.

These studies indicate that protein or lipoprotein might be involved in

hexose carrier function, but although the extracts showed specificity towards optical isomers, they failed to mimic a number of other features of the hexose transport system, for example, saturation and the effects of phloretin.

CONTENTS

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ABBREVIATIONS

ATP	-	adenosine triphosphate
CAM	-	Cellulose Acetate Membrane
CDNB	-	1-chloro-2:4-dinitro-benzene
cpm	-	counts per minute
DMAP	-	2-dimethylamino-ethyl cyanide
dpm	-	disintegrations per minute
EDTA	-	diaminoethanetetra-acetic acid disodium salt
EHT	-	extra high tension
FDNB	-	1-fluoro-2:4-dinitro-benzene
G-M	-	Geiger Muller
NEM	-	N-ethyl maleimide
POPOP	-	1,4-di-2-(5-phenyl oxazolyl)-benzene
PPO	-	2,5-diphenyloxazole
RM	-	reaction mixture
SDS	-	sodium dodecyl sulphate
Temed	-	NNNN-tetramethylethyl - enediamine
Tris	-	tris (hydroxymethyl) methylamine

SECTION 1 INTRODUCTION

GENERAL INTRODUCTION

It was Professor E. Newton Harvey who once wrote 'No one can fail to be impressed with the great difference in properties of living and dead cells. The dead cells are completely permeable to diffusible substances, while the living retain one material and pass another. It can truly be said of living cells that by their membranes ye shall know them'.

At the turn of the century it was evident that the cell was surrounded by a semipermeable membrane. Passage of substances through such a membrane was shown to be dependent on the molecular size of the substance, [Collander, 1924, 1926], and on the lipoid solubility of the substance, [Overton, 1899, 1902 and Collander, 1937]. In a 1917 review, Bayliss added that the membrane may form reversible chemical compounds with the substance to which they are permeable, as a third possibility for the passage of substances through the cell membrane. The whole problem of cell membrane permeability was again reviewed by Davson and Danielli, [1943], and a survey of the necessity to postulate that the membrane contained 'carriers' for the movement of certain substances, (eg. amino acids and sugars), across the cell membrane has been given by Stein, [1967].

Characteristics of Carrier Systems

The binding of the substrate molecules to the membrane component acting as a carrier must be a labile one, either by covalent or hydrogen bonding. The binding reaction is presumed to take place on one side of the membrane, the complex then moves across the membrane where dissociation of the carrier-substrate takes place releasing the substrate molecule on the other side. Carrier molecules are generally considered to have characteristics of high structural specificity including stereochemical specificity, high turnover rates, and a high degree of sensitivity towards certain enzyme and protein

inhibitors. (In the case of carriers in bacteria, the so called 'permeases', induction is also a characteristic). These characteristics bear a very striking resemblance to enzyme catalysed reactions.

Characteristics of Solutes Transported

The solutes transported by carrier mechanisms tend to be hydrophilic substances, substances that are electrically charged and even molecules of large size. Normally these substances would not penetrate natural membranes, [Davson and Danielli, 1943], and if agents are used to block the selective mechanism, penetration is not only reduced but also more or less completely stopped. Thus one is able to postulate that the carrier-substrate complex is more lipoid soluble than the substrate itself, [Rosenberg, 1948 and Wilbrandt, 1960].

The facilitated diffusion of sugars across the red cell membrane illustrates those characteristics which are typical of mediated transport.

HEXOSE TRANSPORT ACROSS THE RED CELL MEMBRANE

The simplicity and availibility of human erythrocytes has made the penetration of the red cell by glucose one of the most investigated carrier systems; see review articles, [Widdas, 1963 and Widdas, 1968]. Widdas, [1954], using the photometric method of Ørskov, [1935], to follow the volume changes in erythrocytes, found Michaelis-Menton type kinetics in terms of a simple mobile carrier could be applied to glucose transport. Wilbrandt, [1954], derived similar equations from fragility experiments, but included rate constants for the binding and dislocation enzymes. Reviews of the development of these kinetics and others have been given by Bowyer, [1957], and more recently by Miller, [1969].

ACCUMULATION

Hexoses penetrate the red cell down a concentration gradient until the

internal concentration becomes equal to the external concentration. The rate of penetration of glucose is $10^3 - 10^4$ times faster than would be expected if the process was by diffusion. Extra energy is not required for this process, but relies on thermal energy for the movement of the carrier-substrate complex. Saturation of the carrier mechanism is observed at high sugar concentration where the maximum flux in one direction is opposed by near maximum flux in the other direction. Thus the rate of entry of sugar is reduced at high concentrations, [Ege, 1919], and is dependent on the intracellular concentration, [LeFevre, 1948].

SPECIFICITY AND COMPETITION

Kozawa, [1914], using erythrocytes from adult mammals, found that only those from primates were permeable to sugars. Widdas, [1955], showed that foetal erythrocytes from pig, rabbit, guinea pigs, sheep and deer all displayed glucose permeabilitity of the same order of magnitude as adult human cells. Wilbrandt, [1938], in rechecking Kozawa's, [1914], work showed that the affinities of monosaccharides for the carrier varied, and the affinity for D and L glucose was markedly different. LeFevre and Davies, [1951], gave as the order of the affinity of various sugars - dextrose, mannose $>$ galactose, xylose, arabinose $>$ sorbose $>$ laevulose, which differs slightly from the order of other authors which has been shown to depend on the concentration of sugars used in the experiments. LeFevre and Davies also showed that glucose, having a high affinity for the carrier, could inhibit sorbose, having a low affinity, but sorbose could not inhibit glucose. Therefore competition between sugars is dependent on their affinity for the carrier.

Rosenberg, [1961], found that substitution of the hydroxyl groups of glucose only resulted in a change in affinity for the carrier. He concluded that the hydroxyls at C1, C2, C3, C4 and C6 were not essential for glucose transport, although C2 was required for intestinal absorption. He suggested that binding of the carrier and substrate probably involved all the

hydroxyl groups. LeFevre, [1961a], made a comparison between the two aldose chair conformations defined as 'CI' and 'IC'. He found a distinct relationship could be made between the affinity constant of hexose penetration and the relative strain found in the chair configuration. The 'CI' form was most favoured where the hydroxyl groups lie equatorial and the carbon-hydrogen bonds axial.

INHIBITION

The inhibition of the glucose transport system can be categorised in a similar manner to enzyme inhibitors.

a. Competitive Inhibitors

These inhibitors reduce the affinity of the substrate for the carrier. If the inhibitor and substrate are together, competition for the carrier will occur, so lowering the affinity of each. Diphenolic compounds have been found to be potent competitive inhibitors of carrier systems. Phloridzin had previously been shown to block the energy supply of cells and inhibit the renal tubule absorption of sugars. Although the red cell does not require energy for glucose transport, phloridzin and its aglycone, phloretin, inhibit glucose transport; the latter being the more potent of the two, [LeFevre 1948, and Wilbrandt, 1947]. An asymmetric membrane was suggested by Wilbrandt, [1954], who showed that although radioactively labelled polyphloretin phosphate did not enter the cell, glucose exits were more inhibited than entries by this compound. Bowyer and Widdas, [1958], showed however that a symmetrical system would give these results because during entries glucose will compete with polyphloretin phosphate, but during exits there is a very low concentration of sugar on the outside so that little or no competition occurs. Other diphenols which inhibit glucose transport are those with oestrogenic activity eg. diethylstilbestrol, [LeFevre, 1961a]. However, related compounds are also effective eg. phenolphthalein, [Forsling and Widdas, 1965 and Prebble and Widdas, 1968].

b. Non-Competitive Reversible Inhibitors

These are characterised not by a reduction in the affinity of a substrate but by an inhibition of the carrier's ability to transport the substrate. This inhibition can be reversed by resuspension of the erythrocytes in inhibitor-free solution or by the addition of anti-inhibitor compounds to the medium. Mercurial compounds are common enzyme poisons and LeFevre, [1948], demonstrated inhibition of hexose transport by mercury ion or p-chloromercuribenzoate, but when glutathionine or cysteine was added inhibition was reversed. Lachrymates, chloropicrin, bromoacetophenone and alkyl isocyanate also inhibit glucose penetration, [Wilbrandt, 1954]. Edsall, [1954], showed that mercurial compounds reacted with -SH groups, (and less readily with NH_2), to form dimers of the protein molecules. Replacing free -SH groups with glutathionine or cysteine restores the enzyme to its monomer form. Lachrymators also react with -SH groups and have been shown by Dixon, [1948], to inhibit hexokinase, specifically attacking the -SH groups rather than the S-S groups.

c. Non-Competitive Irreversible Inhibitors

This group is similar to that of (b) but the inhibitor cannot be removed by washing etc. Fluorodinitrobenzene, (FDNB), and the corresponding chloro-compound, (CDNB), were shown by Bowyer, [1954], to be inhibitors. FDNB has been shown to attack -SH, and NH_2, and phenolic hydroxyl groups. However, Bowyer and Widdas, [1956], showed that neither inhibition of tyrosine with diazo hydroxide nor deamination with mild nitrous acid inhibited glucose transport. They suggested that FDNB acted on the -SH bonds in sites neighbouring the carrier and showed, [Bowyer and Widdas 1958], that the washing of inhibited cells did not restore their glucose transport. It was also noted that glucose activated the rate of reaction of cells with FDNB and this could also be done with other hydrogen bonding agents, for example, urethane and urea, [Bowyer and Widdas, 1958]. Krupka, [1971ab], extending this study showed that the non-penetrable competitive inhibitor

maltose, and the firmly bound inhibitor, phloretin, protected the carrier site against FDNB attack.

N-ethylmaleimide, (NEM), was shown by Dawson and Widdas, [1963], to behave in a similar manner to FDNB, where again -SH groups were thought to be involved. This reaction was shown to be specific because malonamide penetration of the red cell membrane was unaffected by incubation with NEM to inhibit glucose transfer. Tannic acid and Triton X-100 were also shown to be inhibitors, [Hunter, 1964], of glucose, mannose, sorbose and laevulose penetration. Tannic acid is thought to produce new cross linkages in the proteins of the membrane, and Triton X-100 was postulated to increase the hydration of the proteins, so reducing the movement of large molecules.

UPHILL TRANSPORT BY COUNTERFLOW

From the original kinetics of Widdas, [1952], it was predicted that competition between sugars for the carrier could lead to the temporary transfer of one sugar against its concentration gradient. This was first demonstrated on rabbit erythrocytes. It had been shown by Park et al, [1956], that if the metabolism of rabbit erythrocytes is stopped with fluoride, facilitated diffusion of glucose is observed at a rate of one thousandth that of human erythrocytes. Therefore in rabbit erythrocytes a concentration gradient for glucose entry is always maintained by the metabolism of the cell itself. Park et al, [1956], allowed rabbit red cells to equilibrate with xylose for two hours, and glucose was then added to the outside medium. As glucose penetrated the cell, xylose was observed to move in the opposite direction against a concentration gradient until the internal xylose was almost halved. A similar experiment was conducted on human red cells by Rosenberg and Wilbrandt, [1957]. Radioactively labelled glucose was allowed to equilibrate with red cells at $0^{\circ}C$, and uphill transport of labelled sugar could be induced by the addition of unlabelled mannose or glucose. The original level of labelled glucose was restored after the

transport of unlabelled sugar had occurred. This experiment became one of the most important pieces of evidence in favour of the mobile carrier hypothesis. The explanation for this, is that on the outside of the cell there is competition for entry between labelled glucose and mannose, but on the inside there is no competition for the labelled glucose. Therefore more labelled glucose passes out of the cell than enters.

EXCHANGE DIFFUSION

Ussing, [1954], made a requirement for a carrier system that the flux ratios should not equal the concentration ratios. LeFevre and McGuinniss, [1960], designed an experiment to demonstrate this for glucose transport in red cells. They followed the gross equilibrium of glucose across the red cell membrane by chemical measurements, and radioactively labelled glucose was then added to the medium at the same glucose equilibrium concentration, and the entry of the labelled glucose was followed. The reaction was stopped at set times by the addition of ice cold mercuric chloride. They found that the facilitated diffusion of glucose across the red cell membrane had the properties of exchange diffusion. Similar studies made by Britton, [1956 and 1964], confirmed that exchange diffusion took place, but that exchange flux was four times faster than net flux. He postulated that the loaded carrier was more mobile than the free carrier. This asymmetric system has also been proposed by Mawe and Hempling, [1965], and Levine et al, [1965], who suggested ratios of 1:4 and 1:1.8 respectively for the rates of unloaded to loaded carriers.

However, results obtained by the induced counterflow of mannose and galactose by glucose, favoured a simple symmetrical carrier rather than the 'fast complex theory', Miller, [1965].

The variation in parameters of the hexose transport across the red cell membrane, which depended on the experimental method used, was investigated by Miller, [1968].

MODELS FOR THE CARRIER

The kinetic evidence obtained from carrier mediated systems prompted authors to postulate various models for the membrane components involved in the formation of the carrier-substrate complex and its subsequent movement.

MOBILE CARRIER MODELS

These are the simplest models which explain much of the available kinetic information. In the transport mechanism it is proposed that the substrate binds with a membrane component to form a 'carrier-substrate' complex; this complex diffuses across the membrane where the substrate is released to the outside. A model such as this has been described by Widdas, [1954], for the hexose transport system in the human erythrocytes.

Carrier systems normally show specificity for the substrate and this specificity could come from the carrier itself or the binding and dislocation enzymes, as described by Wilbrandt and Rosenberg, [1961]. If the specificity depended on the carrier, this molecule could be too large to diffuse across the membrane although rotation of a large protein macromolecule is a possibility. The carrier and the carrier-substrate complex might be expected to be small or lipophilic to allow diffusion through the hydrocarbon barrier. On the other hand, if the specificity depended on enzymes, themselves hydrophilic, and it was assumed that the carrier-substrate complex could reach the dislocation enzyme by thermal diffusion alone, difficulties could be envisaged unless the complex was restricted to a pore.

PORES

Stein and Danielli, [1956], and Adair, [1956], suggested the presence of 'polar pores' in the membrane to explain the facilitated diffusion of glycerol through the red cell membrane. These polar pores were visualised

as hydrogen bonding components which were stereochemically specific and extended through the thickness of the membrane, permitting polar molecules to pass through rapidly. However, such pores failed to explain uphill transport by counterflow and competitive exchange diffusion.

ENZYMES

The resemblance of carrier substrate reactions to enzyme catalysed reactions has often been used for carrier kinetic evaluation. Authors have therefore introduced enzymes into carrier mechnaisms either a) to alter the substrate molecule, or the membrane, or b) to act as the carrier itself.

a) Alteration of the substrate. Enzymes were added to the mobile carrier hypothesis by Wilbrandt and Rosenberg, [1961], as previously pointed out. Stein, [1962ab], described how dimerisation of two substrate molecules could reduce the number of free hydrophilic groups and allow penetration of the dimer. For specificity, a dimerisation enzyme was included. This model was proposed for the glycerol and glucose transport across the red cell membrane. However, the model had to be discarded because Stein had used inappropiate extrapolation procedures in his kinetic analysis to suggest that more than one substrate molecule was involved, [LeFevre, 1966, and Miller, 1966].

The objection of enzymes situated on both sides of the membrane was overcome by Crane, [1966]. He suggested that if the membrane had a certain degree of flexibility, 'thinning' of the membrane could cause a local interaction of the two protein layers. Thus a specific binding site on the outside of the membrane could be briefly exposed to the cell contents and allow transfer of substrates.

b) Enzymes acting as carriers. The idea of plugs of protein transversing the membrane and acting in the transport of substrate, was first put forward by Mitchell, [1957], as a possible mechanism for the uphill transfer of phosphate ions in respiring bacteria.

Koshland, [1960], redefining the action of enzymes stated that the natural conformation was not the negative of the substrate but that substrates induced a change in conformation which produced a correct alignment of catalytic groups for reaction to occur. A similar approach has allowed Vidaver, [1966], and Stein, [1969], to describe models for glucose transport across the red cell membrane. Vidaver described an allosteric enzyme model, involving transition of a combining site between two alternate shapes, or transition between alternate hydrogen bonding states. He showed that these models could satisfy similar kinetics to the simple mobile model of Widdas, [1952]. Stein, [1969], described a model in which mobile protein subunits with binding sites for glucose were embedded in the membrane but anchored to the aqueous phase. When the subunit was associated with its symmetrical subunit at the opposite face of the membrane, transfer of the sugar would occur. To explain the quantitative predictions of such a model, Lieb and Stein, [1970], modified the protein subunits into the form of a tetramer transversing the membrane. This tetramer could exist in two conformational states. In the normal state (A) binding sites were exposed to the external bathing fluid. After binding there is a substrate induced conformational change (B) where the pair of sites are in communication with each other through an internal pool. In the internal pool redistribution of substrate molecules could occur, internally bound substrate allowing a conformational change back to (A), and the substrate released to the external fluid. Experimentally glucose appeared to have two affinities depending on the conditions, [Miller, 1968]. Therefore Lieb and Stein proposed two types of binding sites; two of the protein subunits binding glucose with a high affinity, and two with a low affinity.

It is necessary to consider how these stereochemical models might be incorporated into the ultrastructure of the membrane.

MODELS OF BIOLOGICAL MEMBRANES RELATED TO PERMEABILITY

BIMOLECULAR LIPID LEAFLET

In 1935 Danielli-Davson presented their 'paucimolecular model' for the ultrastructure of biological membranes. Bragg and Bragg, [1924], and other workers had shown that lipids naturally formed bimolecular leaflets with their hydrophobic tails arranged end to end, and Gorter and Grendel, [1925], suggested that natural membranes contained a bilayer of lipid. Later, based on this evidence, Harvey and Danielli, [1938], added two adsorbed layers of globular proteins to the outer layers of the bimolecular lipid leaflet to account for the low surface tension observed at the surface of cells. This view was further developed by Danielli and Davson, [1943].

Robertson, [1957], introducing his 'unit membrane' as the structure for all biological membranes modified Danielli-Davson's model using the evidence taken mainly from myelin. He suggested that the protein layers were in the β form rather than globular, and that the membrane was asymmetric allowing the outer surfaces to be richer in carbohydrate, (mucopolysaccharides).

The main criticism for this model was that it did not allow for the permeability of certain water soluble substances and small ions through the hydrophobic lipid leaflet. This prompted Stein and Danielli, [1956], to introduce polar pores traversing the membrane but these could not explain all the kinetic features.

There was little doubt that the ability of the cell to act as a barrier to free diffusion rested on the properties of the bimolecular leaflet which indicated that the carrier-substrate complex must be more lipid soluble than the substrate itself. Thus modifications of Robertson's unit membrane to explain special permeabilities and the changes which occur on depolarisation was required.

Watkins, [1965], suggested that alterations in lipid-protein interactions could result in permeability changes. He proposed that the similar structure

of the reacting groups of acetyl choline and the choline contained in lecithin could lead to competition for interaction with proteins of the membrane. The resulting conformational change could possibly account for an increased permeability to Na^+ and K^+.

MICELLAR MODELS

Sjostrand, [1963abc], using potassium permanganate and osmium fixed, or frozen mouse kidney tubule and pancreas for electron microscopy, observed a new repeat substructure in the plane of mitochondrial and smooth surfaced cytoplasmic membranes. This substructure, discussed in terms of lipid globular micelles, was separated by opaque septa which were possibly proteins, [Sjostrand and Elfvin, 1964].

These proteins could be 'structural proteins', [Criddle and Willemot, 1968], and could also contain the enzymes for the electron transport system for mitochondria. From this electron microscoptical evidence, Lucy, [1964], postulated a biological membrane built on lipid micelles. In the plane of the membrane the micelles were visualised as in hexagonal close packing which would allow flexibility and continuous random movement about their mean position. The micelles would have a lipid core of 40°A diameter. Adjacent micelles would be held together by hydrogen bonding, [Lucy and Glauert, 1964], whilst electrostatic interaction would be expected to play a more or less important role depending on the nature of the phospholipid making up the micelle. The stability of the flat sheet of micelles could be accounted for in terms of interaction with extended proteins or mucopolysaccharide molecules on the surface of the lipid.

Fleischer et al, [1963], showed addition of phospholipid to delipidized mitochondria resulted in rebinding of phospholipid and restoration of the electron transfer system. If the membrane was in micellar form this rebinding of protein and phospholipid could mean that the protein penetrated the hydrocarbon chain, looping round the lipid micelle or could mean that the

micelle was made up of lipoprotein molecules rather than just a loose association between lipid and protein. Another indication of stable lipoprotein units has been demonstrated by Gent et al, [1964], who showed that lysolecithin solubilisation of myelin resulted in only one component being formed as shown by electrophoresis and ultracentrifugation.

An inherant feature of the micellar model was that, although the membrane remained lipoid in character, the polar groups of phospholipids and associated counter ions lined pores which had a radius of $4^{o}A$ at their narrowest point. This was in good agreement with the estimated equivalent pore radius of $3.5^{o}A$ - $4.2^{o}A$ from the work of Goldstein and Solomon, [1960], on osmotic pressure measurements from human red cells.

Gammack, [1966], showed using cerebral cortex tissue, that the integrity of the lipid layers was essential for the maintenance of the differential concentrations of sodium and potassium on which the resting and action potentials depended. He suggested that ion selectivity relied on specific orientation and spacing of the polar groups of lipid molecules which might occur in the lining of membrane pores, or by a greater selectivity of lipids in micellar form.

The differences between the permeability of membranes required that the number of pores postulated would have to vary. For example, it would require only one percent of the red blood cells to have micellar configuration to account for its permeability towards water and small ions, whereas myelin would require an even lower proportion. Thus to postulate a micellar organisation and yet explain the different proportions, it is assumed that some of the intermicellar pores could be blocked by 'icelike' structural water, [Hecter, 1965].

Membrane bound enzymes had also been placed in Lucy's micellar membrane. Certain lipid micelles could be replaced by globular protein molecules of the same dimensions. The functional groups and active sites of enzymes would then become an integrated part of the membrane, and also the flexible lattice

of lipid micelle would allow the partial rotation of those protein molecules concerned in the transfer of substrate molecules from one side of the membrane to the other, [Sjostrand, I963a].

REPEATING UNITS AS MODELS

The possible existence of membrane subunit building blocks, partly protein and partly lipid, has also been postulated, [Green and Perdue, I966]. Criddle and Willemot, [I968], showed that extracted 'structural proteins' bound phospholipids hydrophobically, and the work carried out by Blasie et al, [I965], on retinal rod outer segments from frogs using X-Ray diffraction and electron microscopy, demonstrated that there were globular subunits of diameter $40^{o}A$ - $50^{o}A$ making up the plane of the membrane. Wallach and Zahler, [I966], suggested that there were both structural and functional proteins. They envisaged two hydrophilic sections to lie at the membrane surface connected by hydrophobic rods penetrating the membrane. There could be two types of arrangement; single units with non-polar side groups or aggregates in the form of micro tubules which, depending on their primary structure, could have a polar interior.

Membrane transport could occur via these aggregated structures depending on the conformational state of the protein subunit. Stability of this model would depend on hydrophilic bonding to non-penetrating proteins and polar head groups of the lipids. The complex structure of such membrane proteins would depend on their association with appropriate lipids; interactions being hydrophobic.

Current research on membrane models now appears to favour the unit membrane. The evidence for this is based on freeze cleavage techniques which indicate that a continuous hydrophobic space exists, [Branton, I969]. However, to be able to explain permeability it is possible that the membrane utilizes both lipid leaflet and micellar formation and that myelin, [Robertson's

'universal membrane'], being an 'electrical insulator', would show none of the latter. Luzzati and Husson, [1962], pointed out that for some lipoprotein complexes, their ordinary physical condition was not far from the border line of the phase changes from liquid crystalline to coagel. The micellar would represent a liquid interior and the leaflet a crystalline one, [Pethica, 1967]. Possibly if temperature or concentration altered the hydrocarbon chains could crystallise, blocking some physical activity of the lipid and providing a feedback for the restoration to normal conditions.

Eisenman et al, [1968], demonstrated that the moncyclic antibiotic, monactin, acted as a neutral molecular carrier for cations through a phospholipid bilayer. Recently Eisenman has shown that the lipid must be in a liquid state in order for monactin to operate as a carrier.

Recent evidence from freeze cleaving and etching of red cell ghosts, [Weinstein and Koo, 1968, and Branton, 1969], has shown membrane associated particles protruding above the plane of the membrane. There are more of these particles on the outer cleaved surface than the inner, but there is also evidence that some particles penetrate the membrane surface in the form of a cylinder. Possibly these are protein macromolecules or aggregates of protein molecules with a functional role, for example, as a transporting enzyme.

CHARACTERISATION OF THE RED CELL MEMBRANE

The structure of membranes is thus composed of lipids, proteins, a little mucopolysaccharide and in some cases nucleic acids. There is convincing evidence that the hydrocarbon chains of the lipids in natural membranes exist in a liquid crystalline state rather than an immobile solid phase, [Chapman, 1968]. Interaction between protein and lipid is predominantly by hydrophobic bonding, although to a minor extent there is some hydrophilic interaction with lipid headgroups. Protein molecules are generally 100 times larger than the lipids, therefore, providing their tertiary structure

is compact, only a small fraction of their weight is required for interaction. Thus we may have a major class of membrane proteins floating as single molecules or oligomers in a pool of 'liquid' hydrocarbon provided by the lipids.

LIPIDS OF THE ERYTHROCYTE MEMBRANE

Exhaustive studies using recent techniques, such as thin layer chromatography, gas chromatography and infra-red spectroscopy have allowed an accurate assessment of the lipids found within the membrane. The interest in these lipids resides mainly with the quantitative variations between red cells from different animals. Nelson, [1967a], found that the ratio of cholestrol to phospholipid was similar for a number of different mammals, and that cholestrol was the only neutral lipid found. Other neutral lipids, for example, cholesteryl esters, triglycerides and free fatty acids, often seen using infra-red spectroscopy, were probably contaminants and could be removed by strict washing of the stroma. Glycolipids were the only class of lipids that appeared to vary significantly between species. For example, the percentage of total lipids were found to be approximately 26% cholestrol, 50 - 70% phospholipids, and glycolipids varied from 5.3% in rabbit to 23.5% in horse, [Nelson, 1967b].

An accurate analysis of phospholipids by Rouser et al, [1966], gave the following comparative ratio for human erythrocyte; phosphatidyl choline: phosphatidyl ethanolamine: sphingomyelin: phosphatidyl serine of I:I:I:0.5. The only phospholipid seen to vary between species was lecithin which was not found in sheep, cow and goat, [Nelson, 1967b]. This variation prompted authors to speculate whether phospholipid composition could be related to the permeability properties of the membrane. Two groups of animals with differing permeability properties were studied with respect to the phospholipid composition of their erythrocytes, [De Gier et al, 1966]. Man, rabbit, rat and guinea pig red cells haemolyse rapidly in the presence of

glycerol but pig, dog, cat, sheep and cow cells do not. Some correlation was made between the phospholipid content of these two groups except for dog whose cells had a high phospholipid composition but were also permeable to glycerol. van Deenen et al, [1963], found that increasing the linoleic acid in the diets of rabbits made their cells more resistant to haemolysis. However, if the linoleic acid was reduced in the same animal, even though there was a decrease in the incorporation of linoleic acid into the erythrocytes, there appeared to be no reversal of the effect to haemolysis. Walker and Kummerow, [1964], found a distinct relationship could be made between the rate of haemolysis in isotonic solution of non-electrolyte and total fatty acids. By altering the fatty acid composition of the diet, they found that lowering the intake of linoleic acid led to an increased incorporation into the cell of palmitoleic, oleic and eicosatrienoic acid and that the fragility of the cells was increased. They concluded that in red cells, the resistance to haemolysis was due to the high level of polyunsaturated fatty acids.

However, no direct correlation between the permeability properties of red cell membranes and their lipid composition has yet been made.

PROTEINS OF THE ERYTHROCYTE MEMBRANE

Optical Rotatory Dispersion, Circular Dichroism and Infra-red Spectroscopy now indicate that the membrane protein is in helical and random coil conformation and very little in β form, [Maddy and Malcolm, 1965, and Lenard and Singer, 1966]. Although enzyme activities were relatively easy to demonstrate in red cell stroma, the analysis of the membrane protein monomers has been very difficult due to the denaturation of proteins during solubilization.

Enzyme activities were reported to be retained in stromal preparations by Schrier, [1963], who showed that aldolase, glyceraldehyde phosphate dehydrogenase, and acetylcholinesterase activities were all associated with the membrane. The retention of aldolase and glyceraldehyde phosphate dehydrogenase was dependent on pH and osmotic strength, [Mitchell et al, 1965],

whereas acetylcholinesterase was bound more firmly to the membrane but could be solubilized with the membrane lipids using I.2M NaCl, [Mitchell and Hanahan, I966]. Pennel, [I964], in his review of the composition of the red cell membrane, accepts that after haemolysis, a number of enzymes were retained by the membrane, including those factors responsible for sodium and potassium transport. Nilsson and Ronquist, [I969], have isolated a component containing glyceraldehyde 3 phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase from 'white' red cell ghosts using density gradient ultracentrifugation.

Butanol solubilization of salt free stroma has been used by a number of authors to prepare membrane proteins, [Poulik and Lauf, I965, Maddy, I966 and Rega et al, I967]. After centrifugation of the butanol emulsion, the lower aqueous phase is found to contain up to 83% of the membrane protein. Carbohydrates are found to be associated with this protein, but the only lipid present in the aqueous phase is phosphatidyl serine. The protein prepared from the aqueous phase is heterogeneous in character and shows species variations on the analytical ultracentrifuge, [Zwaal and van Deenen, I968a]. A similar protein can be prepared by using n-pentanol, but in this case all the lipids are associated with the protein in the aqueous phase, [Zwaal and van Deenen, I968b].

A lipoglycoprotein released after ether extraction of stroma was termed 'elinin' by Moschowitz and Calvin, [I952]. Elinin contained the Rh and A(B) blood group factors, and the alcohol:ether extraction freed the structural protein 'stromatin'. A similar material was fractionated by Rosenberg and Guidotii, [I969]; the glycoprotein residue left after lipid extraction with ethanol ether (3:I) consisted of 90% of the membrane proteins. This protein had an apparent molecular weight of 60,000 when dissolved in 3% sodium dodecyl sulphate, (SDS), but could be fractionated into five peaks on Sephadex G-200 with I% SDS. Acrylamide gel electrophoresis and N-terminal amino acid analysis demonstrated that the glycoprotein was made up of at least twelve

different polypeptides, present in different amounts and ranging in molecular weights from I0.000 - I50,000. Three of the fractions from the Sephadex G-200 columns showed a higher content of non-polar amino acids than the membrane itself, and although these were heterogeneous in nature, they probably consisted of the 'structural' proteins.

Hot phenol has been used to extract mucoproteins from the red cell membrane which contained immunological activity. Kathan et al, [I96I], extracted an inhibitor of a viral haemagluttanin, of molecular size, 30,000 which also had M and N blood group activity associated with it. Morawiecki, [I964], dissociated this fraction into subunits using the detergents, Teepol or SDS. Removal of the detergent resulted in aggregation of the subunits. Demus and Mehl, [I970], found that phenol, acetic acid and water, solubilized pig erythrocyte membranes into a heterogeneous mixture of monomers. From polyacrylamide electrophoresis the molecular weights of the I4 - I6 zones ranged from 27,000 - I50,000; the major components being 27,000 for I3% of membrane proteins and 48,000 for 30% of the proteins.

Two protein fractions are obtained by using 33% aqueous pyridine, [Copeland and Blumenfeld, I969]. One fraction is water soluble and lipid free, and the other is an insoluble lipoprotein. These fractions show similar multiple zones on gel electrophoresis, demonstrating that the two fractions are related. Although the water soluble protein is associated in the presence of salts it can be resolved on polyacrylamide gel electrophoresis with SDS into twenty protein bands, [Blumenfeld et al, I970].

Less 'harsh' methods of solubilizing the membrane may be carried out using detergents. . The non-ionic detergent, Triton X-I00, was used in conjunction with 8M Urea by Schneidermen, [I965], to solubilize erythrocyte membranes. The protein gave reproducible patterns on acrylamide gel electrophoresis. If solubilization is used with detergent only, the protein extracted can be compared to the structural protein from the mitochondria of neurospora extracted with Triton X-I00. Mazia and Ruby, [I968], found

that if a strict washing procedure to remove ions was followed for the preparation of ghosts, up to 50% of membrane protein was found in aqueous solution after exhaustive dialysis at pH 9.3. However, if Triton X-100 was included in the washing procedure up to 80% of protein would be found in the dialysate. They found this protein to bear similarities to the protein obtained by lipid extraction with butanol.

'Spectrin' and actin like protein has been isolated by Marchesi and Steers [1968], from pink ghosts using exhaustive dialysis of stroma against ATP, (or EDTA), and β mercaptoethanol. Although spectrin does not bind ATP it polymerizes in the presence of divalent ions to form coiled filaments similar to the action of actin, [Steers and Marchesi, 1969]. Spectrin, a homogeneous protein of molecular weight 120,000 - 140,000, was found on the inner surface of the membrane, and showed variation between species. Under mild procedures, such as a reduction in ionic strength at an alkaline pH, about 50% of the membrane protein from 'white' ghosts will be found in solution, [Hoogeveen et al, 1970]. Two protein fractions were obtained, the first, ionically bound to the insoluble membrane, is easily solubilized in water at pH 7; and the second, resembling spectrin, requires solubilization in a salt free environment.

The first fraction was shown to be composed of four major components of molecular weight, 30,000 - 48,000. The residue left after protein extraction contained the lipids, Na^+ and K^+ ATP'ase, cholinesterase, antigenic activity and most of the sialic acid and carbohydrate.

Laico et al, [1970], have demonstrated the presence of a 'mini' protein after solubilization of human and bovine erythrocytes in SDS. It was suggested that this protein, which had been shown to be a major component of the membrane, having a molecular weight of 5,000, might, because of its small size, play a fundamental role in several membrane functions.

A new approach to protein characterisation is the labelling of proteins within the membrane. Berg, [1969], found that if a diazo salt, (^{35}S), sulphonic acid is used on the outside of the red cell membrane, even though

it could not penetrate the cell itself, the cell became impermeable to Na^+ and K^+ but not to water soluble non-electrolytes. Fractionation of the membrane showed that most of the isotope was found on a protein of molecular weight, 140,000. To prevent lysis of the cell, diazotized [^{131}I] diiodosulphanilic acid has been used in a similar manner, [Sears et al, 1971].

Apart from these structural considerations there have been attempts to identify components which might be involved in carrier mediated transports, and these will now be reviewed.

ATTEMPTS AT THE ISOLATION OF THE TRANSPORTING SYSTEM

The type of 'carrier' postulated from kinetic studies for the transport of sugars across the red cell membrane, is presumably either lipid, protein or a combination of the two. Investigations have been carried out to isolate and identify this component.

A number of physicochemical methods have been constructed to demonstrate certain of the properties observed in the hexose transport system. LeFevre et al, [1964], found that a hexane solution, containing crude lipid extract from human red blood cells, could complex with a dried film of glucose. In the presence of the inhibitor, FDNB, the amount of glucose uptake into the hexane solution was reduced. On addition of water, glucose leaves the hexane phase in two stages. Initially there is a fast movement associated with the transfer of phospholipid in the same direction as the glucose, which is followed by a slow transfer of glucose dependent on the occupation of the interface by a layer of phospholipid, [LeFevre, 1967a]. The failure to demonstrate the movement of glucose from an aqueous phase to an oil phase was overcome by Jung et al, [1968], who demonstrated that the solubilization of glucose by chloroform could be enhanced by the addition of phospholipid to the chloroform layer. LeFevre et al, [1968], extended this two phase system into a three phase one, ie. input aqueous phase : chloroform phase : output

aqueous phase. Addition of crude lipid extract from human red blood cells to the chloroform phase greatly accelerated the transfer of glucose from the input to the output side. In such a system, the resistance to movement of glucose was shown to lie at the interfaces, as the glucose gradient across the chloroform phase became very small during the steady discharge of glucose from the input phase. Purification of the crude lipid extract showed that the transfer was due entirely to the phospholipids. All phosphatides accelerated transfer, but within this group, those lipids with an increased polarity tended to be more active. However, unlike the intact human red blood cell, this system did not show similar selectivity between sugars, and saturation of the system was not observed as transfer always depended on the input concentration of glucose.

In contrast to the above, Moore and Schlowsky, [1969], used a stirred two phase system of butanol and water to compare the rates of transfer of D-glucose and D-galactose from the aqueous to the oil phase. In the intact human red blood cell, D-glucose, having a higher affinity for the carrier, penetrates the red cell at low sugar concentrations faster than D-galactose, but slower at high concentrations. In the two phase system, addition of crude lipid extract to the butanol, increases the rate of sugar transfer into the oil phase, and here too at low concentration, (0.015M), D-glucose transfer is faster than D-galactose, but with an increased sugar concentration, (1.3M), D-galactose transfer is faster than D-glucose. If no lipid is added to the oil, D-glucose transfer is faster than D-galactose at all sugar concentrations.

Fractionation of lipid extracts has been used to demonstrate glucose-lipid complex formation. Forsling et al, [1964], extracted lipids from human red cell ghosts incubated with the radioactively labelled inhibitor, FDNB. On a silicic acid column three major labelled peaks were found, two of which were probably lipid-FDNB derivatives eluted with chloroform-methanol 4:1 and 1:4. FDNB is also known to bind unspecifically to proteins, so a similar

study was made using radioactively labelled glucose, [Mawsdley and Widdas, 1967]. A second peak to that of free glucose was observed on the silicic acid column eluted with chloroform-methanol 1:4. A material extracted with pyridine was shown by thin layer chromatography to be a glucose-lipid derivative. However, these materials did not show any specificity for D-glucose when compared with L-glucose.

Another approach in the isolation of a transporting system has been directed towards the proteins of the membrane. Langdon and Sloan, [1967], postulated that the formation of transient imine bonds between the sugars and lysyl residues of the membrane proteins could constitute the carrier.

ie. $R^1CHO + N_2HCH_2R^2 = R^1CH{=}NCH_2R^2$
glucose lysine imine

Incorporation of radioactively labelled glucose could be shown if the imine was reduced to the stable secondary amine by sodium borohydride. Inhibition by phloretin and different affinities of various sugars was shown on this system, and polyacrylamide gel electrophoresis gave a highly labelled zone which was comparable to the migration of L-lysine. From similar studies on non-specific proteins, for example, serum albumin or haemoglobin, it was postulated that proteins showed a low affinity for the sugar, but that the intact human red cells had in addition a relatively small component of much higher apparent affinity, comparable with that for the transport function of these cells. However, LeFevre, [1967b], showed that Langdon and Sloans' results were best equated if only the low affinity sites were considered, assuming the high affinity sites to be zero.

A very interesting approach to the problem involved the column retardation experiments of Bobinski and Stein, [1966], and Bonsall and Hunt, [1966], and Levine and Stein, [1967]. Chromatographic columns were prepared where the stationary phase was composed of membranes or membrane fractions immobilized by adsorption onto celite or diethylaminoethanol-cellulose. These columns were loaded with a mixture of radioactively labelled D-glucose and D-sorbose

(or L-glucose), and the elution of the label was measured. A retardation of the elution pattern of D-glucose compared with the low affinity sugars was observed, which was explained in terms of a specific binding of the D-glucose to the membrane component on the column. However, LeFevre and Masiak, [1970], compared columns prepared by using similar pink ghosts to the above authors, with white ghosts. They showed that the retarded elution patterns observed were entirely dependent on the vesicle space of the membrane making up the column, and that these measurements were of the permeability properties of intact ghost membranes and not specific binding.

LeFevre and Masiak, [1970], also found no evidence for retardation of D-glucose using the membrane extracts of Bonsall and Hunt.

Levine and Stein, [1967], also used ultrafiltration and dialysis techniques to demonstrate specific retention of D-glucose, (compared with L-glucose), by membrane extracts from human red cells.

None of the binding experiments involving model systems, using lipids derived from red cell membranes, show the specificity for substrates which the hexose transfer in intact cells show, and the majority of investigations now conclude that proteins must be involved in some capacity.

THE PRESENT PROBLEM

Before a complete understanding of carrier systems is possible, it is essential to identify those components within the membrane which make up the transporting system. One of the simplest of carrier systems is the facilitated diffusion of glucose across the human red cell membrane. Although the sugar must combine with some compounds of the membrane, the evidence for the nature of these components is far from conclusive.

The object of this thesis was to investigate proteins extracted from human red cell membranes in terms of the role they might play in the transporting system. The approach to the extraction of the membrane proteins was similar to the approach used to show enzyme activity within the membrane. Such

'harsh' methods using phenol, acetic acid or urea to solubilize the membrane could not be used. The starting materials for the extraction of membrane proteins were red cell 'ghosts'. It had been demonstrated by Teorell, [1952], that haemoglobin free erythrocytes still behaved as perfect osmometers allowing the diffusion of water, and also maintaining the Na^+ and K^+ concentration difference observed in intact cells. The facilitated diffusion system of glycerol and glucose was also retained by human red cell ghosts, although the affinity constants were slightly raised, [Stein, 1956, and LeFevre, 1961b].

To obtain a relatively pure protein solution containing a minimal amount of lipid, the n-butanol extraction technique was first used to solubilize the red cell membrane. Rega et al, [1967], reported that this protein had undergone a minimal amount of conformational change. After solubilization there was only a 5% change in p-chloromercuriphenylsulfonate titratable -SH groups, whereas a five fold increase might have been expected if denaturation had occurred. Enzyme activity was also found in this material up to 74 - 107% of the cation independent nucleosidetriphosphatase and the alkaline monoester phosphohydrolase, but the cation dependent enzymes were markedly reduced during solubilization. A little of the firmly bound acetylcholinesterase activity was retained in the protein solution, [Bonsall and Hunt, 1966].

Results from the butanol system indicated that a lipid environment might be important for the glucose transfer system, so a solvent was chosen to form a single phase for the plasma membranes. The detergents are known to be the least harmful of the solvents used for solubilization. The common detergents used are sodium dodecyl sulphate, sodium deoxycholate and Triton X-100, which were compared by Dulaney and Touster, [1970], for the retention of enzyme activity in rat-liver plasma membrane proteins. Phosphodiesterase, alkaline phosphatase and esterase activity were retained after Triton X-100 solubilization, but phosphodiesterase activity was reduced with sodium

deoxycholate and esterase activity inhibited by SDS. The (Na^+ and K^+) dependent ATP'ase activity was also retained after using Triton X-I00 on rat kidney plasma membrane, [Fitzpatrick et al, I969]. Bonsall and Hunt, [I966], reported that up to I30% of acetylcholine esterase activity was retained after solubilizing red cell membranes with Triton X-I00. Consequently, the non-ionic detergent, Triton X-I00 was chosen for the preparation of membrane proteins.

To demonstrate specific glucose binding with respect to the hexose transport system, a criteria was introduced, stating that the proteins should differentiate between the optical enantiomers. Glucose binding experiments were then conducted against controls containing L-glucose, although the early work used enzyme inhibitors.

SECTION 2 METHODS AND MATERIALS

After a number of experiments had been completed certain results indicated that the protein extracts were contaminated with short rod bacteria. This contamination originated in the buffer solution used in the preparation of the ghosts. It therefore became necessary to sterilise all solutions used in the preparation of ghosts, in the extraction of proteins and for the final experiments. This sterilisation was carried out in a steam autoclave at 15 lbs. pressure, 120°C for twenty minutes.

PREPARATION OF ERYTHROCYTE GHOSTS

Ghosts were prepared from recently outdated human blood, (435ml human blood in acid citrate dextrose). Although no specific blood group was used for the experiments, care was taken to try to obtain blood of only three weeks old. The red cells were initially packed by centrifugation at 3,000 rpm for 20 minutes and the plasma and buffy coat removed with a pasteur pipette attached to a vacuum pump. The remaining red cells were washed three times with 1% saline; the final washing leaving the supernatant clear and colourless.

Dodge et al, [1963], introduced a standard method of preparation of white ghosts. This method was modified by Rega et al, [1967], to prepare haemoglobin free ghosts, and at the same time to ensure a minimal loss of membrane protein. 100ml of washed packed red cells were subjected to osmotic lysis in five stages as follows. The cells were suspended once in 1mM EDTA, 20mM NaCl, 10mM Na_2HPO_4 - KH_2PO_4 (pH 7.4) for 60 minutes and four times in 10mM NaCl, 5mM Na_2HPO_4 (pH 7.4) for 15 minutes. EDTA is included in the first lysis to release the haemoglobin bound to the membrane by heavy metals. The cells or ghosts were exposed to eight times their volume in each case, and recovered by ultracentrifugation at 15,000g, 4°C, for 20 minutes. A red button of cells, which adhered to the tube, was

discarded from the ghosts. In some cases not all the free haemoglobin was removed, so these cells were not used in the experiments. In later preparations the phosphate buffers were replaced by Tris-HCl, which allowed a more reproducible haemolysis.

In all centrifugal procedures polyethylene tubes were used, which could easily be sterilised using 70% absolute alcohol for five minutes. To prevent contamination during the transferring of the ghosts for different experiments, the ghosts were divided up into 25ml sterilised McCartney screw top bottles. Usually one bottle was used per experiment.

EXTRACTION OF THE PROTEIN

PROTEIN SOLUBILIZED BY BUTANOL EXTRACTION

To obtain a reproducible protein extract, very careful procedures had to be followed. The method chosen was essentially the one described by Maddy, [1966], and Rega et al, [1967]. Maddy noted that to obtain a butanol emulsion it was necessary to have a salt free environment. Ghosts were washed three times with chilled deionised distilled water by suspending the ghosts in four times their volume, and allowing them to stand in a refrigerator for five minutes before ultracentrifugation at 15,000g, 4°C. 15ml ice cold n-butanol was added to 30ml of the water washed stroma, (each ml containing 6-8mg dry wt), in a 50ml polyethylene centrifuge tube. The contents of the tube were shaken vigorously for 20 seconds and allowed to stand in an ice bucket, and after 15 minutes the white emulsion was centrifuged at 15,000g for 20 minutes. Centrifugation resolved the emulsion into an upper butanol phase and a lower aqueous phase separated by a thick white emulsion. The aqueous phase was carefully removed using an automatic syringe. To the butanol phase and interface, 15ml chilled deionised distilled water was added and shaken vigorously for 20 seconds. The tube was allowed to stand for 15 minutes and then centrifuged at 35,000g which again resolved the emulsion

into an upper butanol and lower aqueous phase. Rega et al, [1967], reported finding a thin insoluble interface at this stage, which was often found in these experiments, although on a number of occasions complete solubilization appeared to have taken place. The two aqueous layers containing the stromal proteins were pooled and stored in sterilised McCartney bottles at $0^{o}C$. During the entire preparation of protein the temperature was kept as low as possible, $0-4^{o}C$. Before this protein could be used in experiments, it was found necessary to remove the butanol solubilized in the water. A number of methods were attempted but the most satisfactory method was rotatory evaporation. The aqueous phase was reduced to half volume by evaporation at $10^{o}C$ under reduced pressure. An equal volume of chilled deionised distilled water was added and the process repeated. This was carried out three times until the smell of butanol had disappeared. Evaporation was continued until the required concentration of protein had been obtained. Depending on the experiment, the required concentrated buffer was added to the solution protein, to give the final concentration of buffer used in the experiments.

PROTEIN SOLUBILIZED BY DETERGENT

In contrast to the above procedure, solubilization of plasma membranes using detergents was comparatively simple and short. Impurities from Triton X-100 were removed by stirring with 10%, (w/v), of coarse silica gel for 20 minutes. After standing for 15 minutes and then filtering through glass wool, Triton X-100 was made 10%, (w/v), with buffer solution, as earlier experiments had shown that Triton X-100 in water was acidic. This Triton X-100, (10%), solution was then slowly mixed with a suspension of ghosts, (containing 1 - 2 mg protein/ml), until the final concentration of detergent was 1%. The polyethylene tube containing the mixture was allowed to stand for 15 minutes in an ice bucket and then centrifuged for 30 minutes at 40,000g. The protein solution could be decanted off leaving a white

residue adhering to the tube. Further extraction of protein from the residue could be carried out by addition of Triton X-100, but this was not found necessary for these experiments. The protein solution was stored in a similar manner to the proteins solubilized by butanol extraction above.

CHROMATOGRAPHY AND ELECTROPHORESIS

The two most valuable techniques used in the fractionation of proteins has been dextran gel chromatography and electrophoresis. In this work both these techniques were used for a dual purpose; primarily to identify a component which binds glucose, but also to make a simple study of the polypeptide units extracted from the membrane.

DEXTRAN GELS

Sephadex is a cross-linked dextran which swells considerably in water and electrolyte solutions and may be used for column chromatography. Different Sephadex gels have different degrees of cross-linkage in the polysaccharide which determines the porosity of the gel and hence its fractionation range. A low degree of cross-linkage produces a gel with large pores, and conversely a high degree of cross-linkage produces small pores and low water regain. When an aqueous solution of large or small molecules filters through a column packed with Sephadex, molecules that are unable to penetrate the gel particles, move faster than the small molecules, which diffuse into the gel grains to varying extents according to their size. The different molecules therefore appear in the eluant in a sequence of decreasing molecular size.

Membrane proteins tend to be associated, and of large molecular size ranging from 10,000 - 150,000 in molecular weight; the smallest structural proteins observed from red cells being 5,000. Sephadex gels have been classified according to their fractionation range using well defined dextran fractions. This range can be increased when considering globular proteins.

Hence, Sephadex G-200, fractionation range 1,000 - 200,000 (5,000 - 800,000 for globular proteins), and G-75, range 1,000 - 50,000 (3,000 - 70,000 for globular proteins), were used for column chromatography.

Gel Chromatography

The columns used were of the glass quickfit type, each equipped with a sintered glass filter. To prevent clogging with Sephadex grains the filter was covered with a thin layer of glass beads. A number of methods of packing the column were suggested by Pharmacia and the one chosen was found relatively simple and produced reproducible columns. Sufficient gel for the required column was calculated on the basis that 3g of G-200 swells to give 100ml and 7g of G-75 gives 100ml of swollen gel. The dry Sephadex was allowed to stand in excess deionised distilled water for three days with intermittent stirring and decantation. In the final washing the water was replaced by the eluant buffer.

The column was clamped in a vertical position (using a spirit level), and sufficiently extended with quickfit adaptors to allow the entire prepared gel slurry to be poured out. To reduce the head of pressure on the column, a narrow polythene tube was connected to the outlet and was clamped up at a position approximately 10cm below the top of the column. One third of the column was filled with buffer, and extra buffer was added to the slurry to prevent air bubbles forming. The homogeneous slurry was then carefully poured into the column and as soon as a layer, a few centimeters thick, had formed, the outlet was opened. As the gel settled, the outlet tube was lowered to keep it 10cm below the surface of the buffer. When the buffer level reached the top of the column the polythene outlet tube was removed and the quickfit adaptors replaced by a constant head Mariotte flask containing buffer. This reservoir bottle was positioned in order to give the exact rate of flow required. Buffer was allowed to pass through the column before the sample was added.

Prior to charging the column, the reservoir bottle was disconnected and

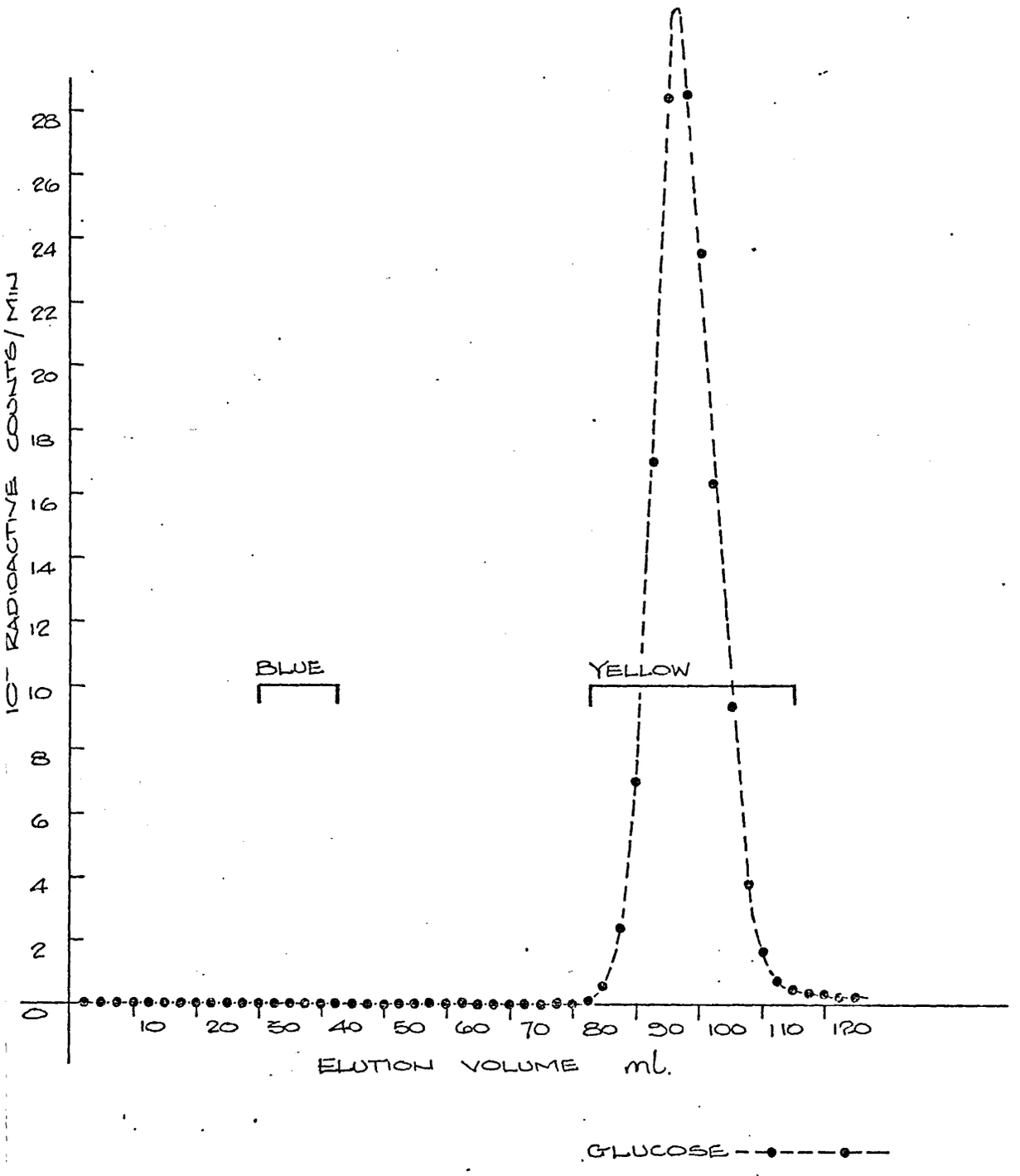


Figure 1 Sephadex G-200 column chromatography (30 x 2cm)

Sample: D-(^{14}C) glucose, Blue Dextran (blue), potassium dichromate (yellow)

Buffer: 150mM NaCl, 10mM Na_2HPO_4-KH_2PO_4 (pH 7.4)

Flow rate: 7.5ml/hour

a large part of the buffer above the gel surface was removed by suction. To prevent disturbing the gel, a circle of Whatman No. 1 filter paper was then allowed to settle onto the surface of the gel bed. Using a pipette, the sample, approximately 1ml of a 1% protein solution, was carefully layered above the filter paper beneath 1ml of buffer. The sample was allowed to penetrate the gel bed and 1ml of buffer was then added. After this had penetrated the gel, the column was filled with buffer and the reservoir bottle reconnected. Samples were collected using a fraction collector incorporating a 2.5 or 3ml siphon dispenser.

Before protein extracts were added, the columns were tested and standardised using a mixture of Blue Dextran, (Molecular weight = 200,000), potassium dichromate and radioactively labelled glucose, (Figure 1). Columns could be used again after each experiment as recharging is automatic during elution. If the column was dismantled, the swollen gel was kept in the refrigerator and sodium azide, (0.02g/100ml), was added to prevent microbial growth.

(Note: Details of the size and dimensions of the Sephadex columns used are given in the results section).

ELECTROPHORESIS

The separation of protein subunits by electrophoresis was first developed by Tiselius, [1937], and was based on the principle that a charged ion, (or group), will migrate towards one of the electrodes when placed in an electric field. Amino acids and proteins, being ampholytes, will migrate to the anode at pH's above their isoelectric point and in the reverse direction at a pH below this. When two electrodes are inserted into each end of a U tube containing a dilute mixture of proteins, migration of each protein will depend on its own characteristic surface electric charge. However, separation of the proteins would not occur as the proteins are originally present throughout the whole tube. To overcome this problem the mixture of proteins was placed

within a narrow band or zone at a suitable distance between the two electrodes. As migration occurs, the different components, which move at different rates, slowly draw away from each other to produce a separation in the direction of migration. This 'zone electrophoresis' is not normally carried out in free solution but the protein is held in either a stabilising gel or paper medium.

The value of buffers (in a continuous system) is to control the pH and to ensure that each component will maintain a constant charge throughout electrophoresis. As the concentration of buffer increases, the components move more slowly due to the greater proportion of charge carried by the buffer ions. Migration is reduced further by the ions, surrounded by buffer ions of opposite charge and moving in the opposite direction, which produces a tighter zone. In the discontinuous system, the electrode buffer will be at a higher concentration than the medium and possibly of a different pH, or a different buffer is chosen for the electrode compartments than that for the gel. The effect of this creates a voltage discontinuity at the interfaces of the two buffers. As the interface travels through the protein band, the band compacts to produce a tighter zone.

Glucose having no charge does not migrate in an electric field. This enabled standard electrophoretic techniques to be modified so that a mixture of proteins could be passed across a zone of glucose. Either retardation of specific proteins, or the movement of glucose under the influence of proteins, could then be measured. The two techniques used were Cellulose Acetate Membrane and acrylamide flat gel electrophoresis. A third technique, acrylamide gel disc electrophoresis, was also used to study protein extracts previously loaded with glucose.

Cellulose Acetate Membrane Electrophoresis

A simple rapid method was required for preliminary investigations. The Cellulose Acetate Membrane, (CAM), was chosen in preference to paper because absorption of the sample is negligible. The membrane is also relatively pure, having a low heavy metal content, and is suitable for glycoproteins,

[Korn, 1968]. Although this technique is not generally used to study membrane proteins, sufficient evidence has shown that it is ideal for the study of serum proteins, [Korn, 1968]. This technique provided a simple method of introducing a zone of glucose in the pathway of the migrating protein. One disadvantage of all paper electrophoresis is the phenomena of electro-osmosis, which occurs when paper and water come into contact. During electrophoresis with an alkaline buffer, the water becomes positively charged with respect to the paper. As the paper is fixed, the water is free to move towards the cathode carrying buffers and neutral components. Glucose then moves to the cathode while the protein migrates to the anode. Preliminary studies were made using blue dextran to estimate the extent of electro-osmosis. This enabled suitable positions on the membrane to be marked for protein and glucose so that during electrophoresis the two would cross.

The method used for CAM electrophoresis was the same as the one suggested by Korn, [1968], for serum proteins. Impregnation of the membrane, (12.5 x 2.5cm), was carried out in 0.07M Barbitone buffer, (pH 8.6). The membrane was allowed to float on the surface of the buffer and to avoid trapping air bubbles, the buffer was allowed to soak through the membrane before it was immersed. After 30 minutes the membrane was removed from the buffer, lightly blotted and laid across the filter paper pads in the Shandon Universal Electrophoretic tank. The filter paper pads act as wicks connecting the membrane to the electrode compartments containing 0.06M Barbitone buffer, (pH 8.6). Before the samples were added to the membrane a preliminary voltage of 40v was passed across the membrane for about 30 minutes. 5µl of sample containing 300µg of protein was 'streaked' approximately one third of the distance from the cathode and 5µl of radioactively labelled glucose was streaked one third of the distance from the opposite end. Electrophoresis was carried out at 0.4ma/cm width of strip for two hours. The membrane was dried, scanned for radioactivity and stained with Nigrosin.

Gelatine covered membranes, (Cellogel), were also used in a similar manner. Because of the gel covering, care had to be taken to prevent the drying out of the membrane. Also as the gel reduced the electro-osmotic effect, glucose was streaked mid-way between the two electrodes.

Acrylamide Electrophoresis

Electrophoresis, using cellulose acetate paper, did not resolve the protein into any of its subunits, although interesting effects were observed with respect to the addition of the zone of glucose. . It was therefore necessary to use the more elaborate gel electrophoresis. The two most popular gels used are starch and acrylamide, which are especially valuable for high molecular weight fractions. Acrylamide was chosen due to the ease of preparation, the relative inertness of the gel, (starch possibly having some effect on glucose), and the rapidity of the electrophoresis. Acrylamide gel was first used as vertical disc electrophoresis by Orstein, [1964], and this is still the most favoured system for the characterisation of membrane proteins.

Gel disc electrophoresis was used to study protein extracts loaded with glucose, but a horizontal flat gel enabled a similar study to the CAM electrophoresis to be performed. In gel electrophoresis, the gel can be considered as a porous medium in which the pore size is of the same order as the protein molecule. A molecular sieving effect is impressed on the normal protein separation, so molecules of similar charge to mass ratio, but with different molecular size, can be separated. Acrylamide can be prepared so that the pore size is variable, ie from 3 - 30%. Increased gel concentration reduces the pore size, and excludes molecules of diminishing size from the gel. Large pore size gels were prepared, ie. from 5 - 10%. as the components of the membrane extracts were expected to be of large molecular size and probably associated. Polyacrylamide gel is prepared by polymerising the acrylamide monomer, (CH_2=$CHCONH_2$), with the cross linker or comonomer, methylenibisacrylamide. Polymerisation requires a catalyst, freshly prepared ammonium persulphate, and an initiator, tetramethylethylenediamine, (Temed), or

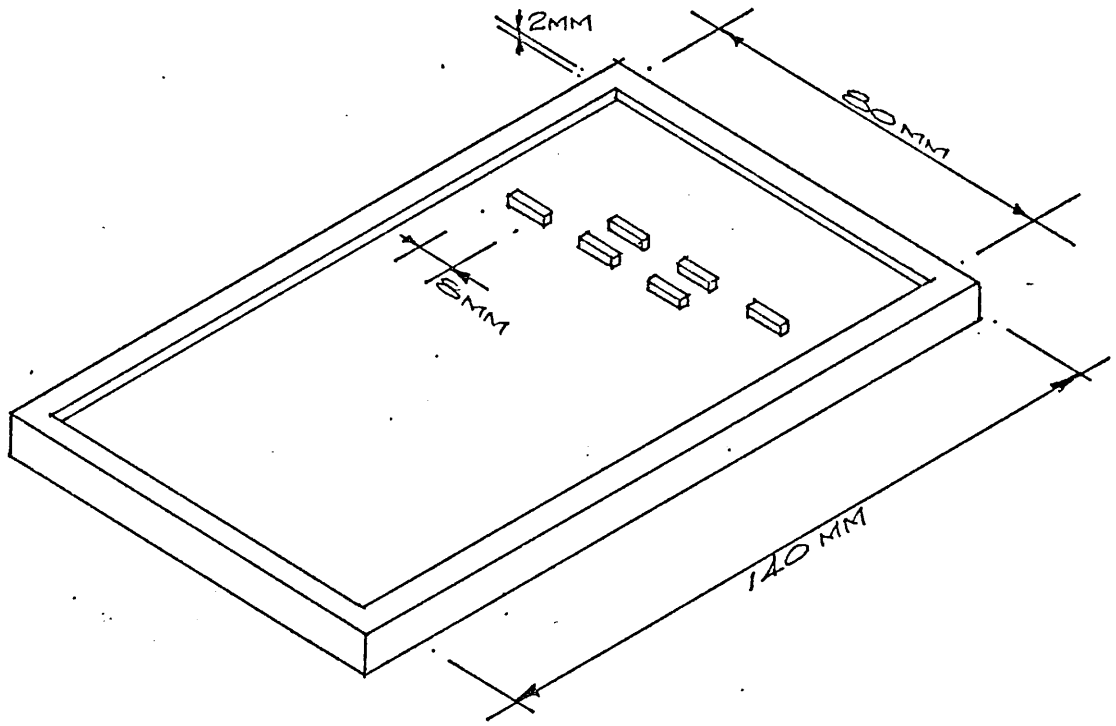


Figure 2 Gel former (without lid) showing the positioning of the sample slots.

dimethylaminopropionitrile, (DMAP). Care is taken during polymerisation to exclude air, as oxygen inhibits the reaction.

a. Flat Gel Acrylamide Electrophoresis

The method used is the one described by Tombs, [1968]. The gel is prepared in water and poured into a 'gel former', and after polymerisation the gel is removed, and the buffer introduced by dialysis. The gel is then placed in a 'gel runner' for electrophoresis. After early experiments it was found unnecessary to prepare the gel in water as it could be polymerised just as easily using the required buffer, thus avoiding the dialysis step. The 'gel former', as represented in Figure 2, is made of perspex. The 'gel runner' is also made of perspex and is of the same dimensions as the former, excluding the perspex slot formers. The double row of slot formers, 0.5cm apart, allow the proteins to pass through a zone of glucose. The dimensions of the slot formers are 8 x 0.5 x 1.5mm allowing 0.5mm of gel at the bottom of the slot to prevent diffusion of the sample. The slot formers were only temporarily fixed so that samples could be placed in different positions, the most satisfactory of which were found to be 3.0cm (for protein) and 3.5cm (for glucose) from the cathode end.

Cyanagum 41, a mixture of monomer and comonomer, was dissolved in water at the required concentration, ie. 5g/100ml for a 5% gel. This solution was filtered using a Whatman No. 1 filter paper before the addition of 0.5ml of DMAP and 1ml of freshly prepared ammonium persulphate, [10% (w/v) in water]. 50ml of this mixture was poured into the gel former and a glass lid was carefully placed on top to avoid trapping air bubbles. After polymerisation of the gel, which normally took about 20 minutes, the gel was removed and transferred to 500ml of gel buffer. Dialysis against gel buffer was carried out overnight with one change of the buffer. The gel was carefully transferred to the gel runner, trimmed to size and blotted with filter paper. Extreme care was taken to remove all the buffer remaining in the sample slots. The gel runner was laid on the bridges of a Shandon Universal Electrophoretic

tank and filter paper wicks, (a double thickness of Whatman 3MM) , placed overlapping the gel by 1cm, and dipping into the electrode compartments. 10µl samples, which were carefully pipetted into the sample slots, contained either 3% protein or ^{14}C labelled glucose. Details of the electrophoretic times and buffers used are given in the results section. After electrophoresis the gels were scanned for radioactivity and then stained with Naphthalene Black. During scanning for radioactivity the gel was covered with a thin perspex lid, in which a longitudinal slit isolated the required sample.

b. Acrylamide Gel Disc Electrophoresis

Unlike the above, the gel was polymerised in narrow glass tubes, 10cm in length and with an internal diameter of 6mm. The gels used by Ornstein, [1964], consisted of the small pore gel, the spacer gel and the sample gel. The sample gel was omitted in these experiments and the spacer gel was formed by the addition of a few drops of water during the polymerisation of the small pore gel. The tubes containing the gels were placed in a vertical apparatus in order to connect an upper and lower electrode compartment. A number of authors, [Shapiro et al, 1967, Weber and Osborn, 1969, and Dulaney and Touster, 1970], have shown that proteins solubilized by detergents are better resolved on acrylamide if the gel is polymerised in the presence of the detergent. Shapiro et al, [1967], reported that the separation of proteins by polyacrylamide electrophoresis in the presence of the anionic detergent, SDS, was dependent on the molecular weights of the polypeptide chains. This was confirmed for forty proteins ranging in molecular weights from 11,000 - 200,000 by Weber and Osborn, [1969].

The procedure of Weber and Osborn was followed in the preparation of the gels. Proteins were incubated for two hours in 10mM phosphate buffer, (pH 7.0), 1% SDS and 1% β mercaptoethanol. Preparation of gels were made from stock solutions; the stock gel buffer consisting of 0.2M phosphate buffer, 0.2% SDS, and the stock gel solution of 22.2g of the monomer plus 0.3g of the

comonomer dissolved in 100ml water. For the preparation of 10% gels with half cross linker, 13.5ml of gel solution was deaerated with 15ml of gel buffer. 1.5ml of freshly prepared ammonium persulphate was added, (15mg/ml), followed by 0.045ml of Temed. The tubes, which had previously been cleaned in nitric acid and then siliconised, were placed in a test tube rack with tightly fitting caps at their lower ends. 2ml of the gel mixture was added to each tube, using a pasteur pipette, and care was taken to ensure that all air bubbles were excluded. As the gel hardened, 20µl of water was layered on the surface of each gel. After 20 minutes the water was sucked off the gels, and the caps carefully taken off the tubes. Eight tubes were then placed in the electrophoretic apparatus.

100µl of sample, (containing approximately 100µg of protein, 1 drop of Bromophenol Blue [1%], 5µl of mercaptoethanol and 1 drop of glycerol), were layered on the gel surface. Gel buffer, diluted 1:1 with water, was then added to each electrode compartment so that the height in each compartment was the same. Electrophoresis was conducted at 2ma per gel for one hour, and then increased to 8ma per gel for a further five hours with the positive electrode in the lower compartment.

Triton X-100 was also used in place of SDS to study the proteins which had previously been extracted from the red cells by Triton X-100. This allowed the proteins to be studied in a constant environment. When Triton X-100 was used, the stock gel buffer consisted of 0.2M Tris:HCl, (pH 8.5) and 0.2% Triton X-100. 7% gels with half cross linker were prepared using 9.45ml of the stock gel solution. 4.05ml of water and 15ml of buffer. Due to the low concentration of the gels, the gels had to be supported in their tubes using caps with their centres cut out. The 100µl samples used for these experiments contained 100µg of protein, made 10% (w/v) with sucrose, plus 1 drop of bromophenol blue. Electrophoresis was carried out at 2ma per gel for one hour, increasing to 3ma per gel for a further four hours.

After electrophoresis, the gels were removed from their tubes by squirting

water from a syringe between the gel and the glass wall, and using a pipette bulb to exert pressure. Gels were either taken for radioactive assay or fixed overnight in 10% salicylic acid and stained with coomassie blue. For radioactive counting the gels were cut up into 5mm segments. Each segment was placed in a screw top glass vial containing 0.1ml of 30% H_2O_2. The vial was then placed in an oven at 50°C overnight to allow the gel to solubilize, [Tishler and Epstein, 1968]. 0.5ml of Packard solubilizer and 10ml of phosphor were then added for radioactive counting. Weber and Osborn had shown that for SDS gels the mobility, which was equal to:

$$\frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

could be plotted against the logarithm of known polypeptide chain molecular weights. Hence measurements of the gels before and after staining were made for the SDS gels.

DIALYSIS

A semipermeable membrane, such as Visking tubing, allows the movement of small molecules but retains large ones such as proteins. Hence, substances of small molecular size may be removed from protein solutions by placing the solution in a visking tube and stirring it against a large volume of distilled water. Visking tubing can also be used to concentrate proteins if a positive pressure is applied on the protein solution in the tube. Levine and Stein, [1967], showed how such ultrafiltration could be used to demonstrate the binding of D-glucose to proteins in solution. In their paper they also reported that dialysis experiments would give similar results.

Dialysis experiments were conducted to try to demonstrate that membrane proteins would retain D-glucose by specific binding within a 'visking tube' bag. Visking tubing, (1 inch wide), was allowed to soak overnight in water, and the short length of tube used was knotted at one end. 1ml of protein solution, previously loaded with part radioactively labelled glucose, was placed in the bag and the free end tied. The bag was placed in a stoppered

conical flask containing 100ml of buffer, including part radioactively labelled glucose. Care was taken to ensure that the radioactive counts and the concentration of glucose were the same in both the internal and external solutions. The flask was placed in an ice bucket and dialysis was carried out in a cold room by slowly stirring the dialysis bag with a magnetic stirrer. A time course for dialysis was obtained by dialysing a number of similarly prepared bags, and removing samples at set intervals.

DOUBLE LABELLING TECHNIQUES

When the Liquid Scintillation Spectrometer became available, double labelling allowed two binding experiments to be devised. The first to study the insoluble interface observed during butanol extraction, and the second to study the total protein solubilized by Triton X-100.

MILLIPORE FILTERING

This method of filtration allows the filtering of very fine particles. Millipore filters are very accurately graded and can be obtained with a pore size of very small dimensions.

Rega et al, [1967], and Zwaal and van Deenen, [1968a], reported that an insoluble interface was observed during the butanol extraction of red cell membranes. Experiments were carried out to collect this interface on a millipore filter and demonstrate binding of D-glucose using a similar method to the one described by Eichholz et al, [1969].

4ml of water washed stroma were loaded with a mixture of radioactively labelled D-(^{3}H) glucose and L-(^{14}C) glucose in a polyethylene tube. Non-radioactive glucose was then added so that the total concentration of D-glucose and L-glucose were the same. After 5 minutes, 2ml of cold n-butanol were added and the tube shaken vigorously for 20 seconds. The tube was placed in an ice bucket for 15 minutes and 1ml samples were rapidly passed through a Versapore, (1.2μ), filter using a swinney adaptor, attached to a 5ml glass syringe. It was necessary to wash the filter immediately, but as water

removed all the glucose on the filter, a non-polar solvent was used. As the Versapore filter resists solubilization by non-polar solvents, 1ml of cold acetone could be used to wash the filter. Radioactive counts were taken of the emulsion, filtrate and acetone washings. The filter was allowed to dry under a lamp and then immersed in 20ml of phosphor for 24 hours prior to counting.

AMMONIUM SULPHATE PRECIPITATION

The solubility of a protein in an aqueous solution is dependent on the concentration of the salts present. Salts in a low concentration tend to aid the solubilization of proteins by coming between the opposing charges on the proteins and thus shielding the protein molecules from each other. In high concentrations, precipitation of the protein might occur by 'dehydration' of the protein molecules. The solvent becomes organised about the salt ions to such a degree that its normal organisation around the protein is decreased and the proteins are able to associate in a solid phase. Precipitation of proteins by increasing the salt concentration, using for example ammonium sulphate, is often used to purify proteins due to the minimal amount of denaturation that occurs.

A simple experiment was used to examine whether the proteins solubilized by Triton X-100 would bind glucose whilst they were being precipitated by ammonium sulphate. Using a saturated solution of ammonium sulphate, a 1:1 dilution was found to precipitate up to 90% of the membrane proteins. Green and Hughes, [1966], reported that 31.1g of solid ammonium sulphate per 100ml would give a 50% saturated solution.

White ghosts were loaded with D-(^{3}H) glucose and L-(^{14}C) glucose plus unlabelled sugars to ensure that the concentration of the two sugars would be the same. The ghosts were solubilized with Triton X-100 in the usual manner. The centrifuged protein solution was placed in a polyethylene tube and cooled in an ice bucket. Solid ammonium sulphate was slowly stirred into the tube

over a period of one hour until a final saturation of 50% was reached. The protein precipitate was allowed to flocculate for 10 minutes and then centrifuged at 15,000g for 15 minutes. After centrifugation the supernatant was carefully removed from beneath the precipitate using an automatic syringe. The excess fluid was allowed to drain from the tube. The precipitate adhering to the side of the tube was then solubilized with 1% Triton X-100. Samples were taken from the reaction mixture, supernatant and solubilized precipitate for radioactive counting.

Determination of the Counts Bound

The number of bound counts can be determined from the distribution of D-(^{3}H) glucose and L-(^{14}C) glucose in each sample and the original reaction mixture. The ratio of glucose in each sample, (Ds/Ls), and the reaction mixture, (Drm/Lrm), is calculated. These ratios give the expected counts for D-glucose, (X), using L-glucose as a control to correct for non-specific binding and entrapment.

$$\text{eg.} \quad \frac{Drm}{Lrm} = \frac{X}{Ls}$$

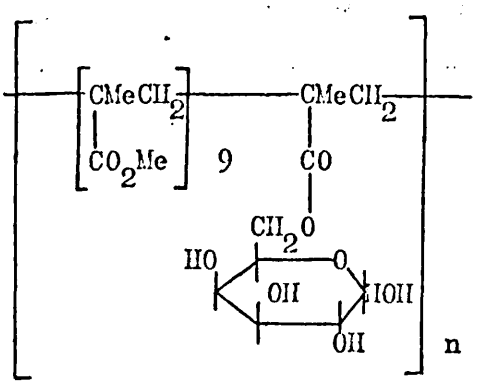
Subtracting X from the total D-glucose in the sample gives the D-glucose counts specifically bound.

POLYMER STUDIES

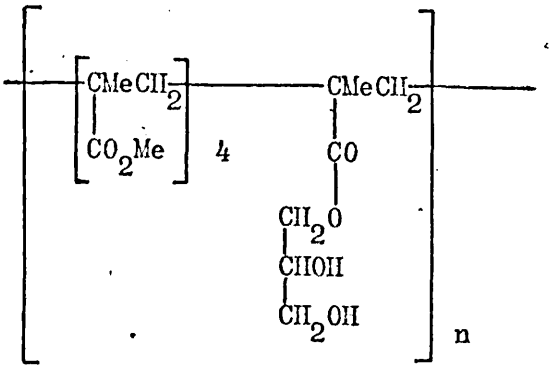
The greatest difficulty in trying to identify the transporting system is that it probably represents only a very small weight of the total membrane. A tentative investigation was therefore carried out to extract specific proteins from the protein extracts. A number of polymers have been prepared using carbohydrates as side groups, [Colquhoun and Dewar, 1968 and Black et al, 1969]. Two methyl methacrylate polymers were obtained with galactose and glycerol side groups, as shown below.

A weighed sample of the powdered polymer was slowly stirred with a dilute protein solution. After 30 minutes the solution was filtered and a sample taken for protein estimation. The polymer was washed rapidly with water and

then allowed to mix slowly for 10 minutes with 10mM glucose. Samples were taken for protein estimation.



methyl methacrylate-galactose methacrylate



methyl methacrylate-glyceryl methacrylate

ASSAY METHODS

RADIOACTIVE ASSAY

Geiger-Muller Counters

a. 50µl or 100µl of sample were carefully pipetted onto 2.5cm aluminium planchets and allowed to dry slowly under a lamp. To produce an evenly spread film across the planchet, a drop of dilute detergent was sometimes added. Duplicate planchets were counted for at least ten minutes, on a G-M counter equipped for anticoincidence counting and giving background counts of 2/minute.

b. Radioactive scanning was performed by placing the cellulose acetate paper or acrylamide flat gel on a trolly moving under the narrow window of a G-M counter. Pulse rates were recorded by an Ecko Ratemeter and the output fed to a pen recorder, in which the paper moved at the same rate as the trolly.

Liquid Scintillation Counter

A scintillator is a substance which emits a weak light flash or scintillation of short duration whenever it is struck by an ionising particle. The organic liquid scintillators normally used, consist of one or more fluorescent aromatic solutes in an aromatic solvent. As the radioactive isotopes used were in aqueous solution the scintillator had to include

substances miscible with water and the aromatic solvent.

The scintillator of phosphor chosen for ^{14}C counting was of the emulsion type described by Patterson and Green, [1965]. 500ml of Triton X-100, with the impurities removed as before, was mixed with 1L of Toluene containing 4g of PPO and 0.1g of POPOP.

The radioactive sample, usually in 100µl of aqueous solution, was shaken with 10ml of phosphor in a glass stoppered vial until a clear solution was formed. The vial was allowed to cool prior to counting in the Panax SC-LP. Calibration of the counter showed that the highest counting efficiency for ^{14}C in the Triton X-100 phosphor could be obtained using E.H.T. volts = 1,500 and Discriminator volts = 8. This gave a background count of approximately 8/minute. The counting for each sample was continued for at least 10 minutes.

Liquid Scintillation Spectrometer

An important property of scintillation counters is that they are able to distinguish between ionizing particles of different energies. The pulse amplitude is proportional to the scintillation intensity. In the case for beta particle irradiation the scintillation intensity is proportional to the energy of the beta particle which produced it. Therefore, as the pulse amplitude is proportional to the beta-particle energy, the scintillation counter functions as a beta-spectrometer. The instrument used for the detection of D-(^{3}H) glucose and L-(^{14}C) glucose was the Tricarb 3 channel Liquid Scintillation Spectrometer. The channels chosen were the $^{3}H(^{14}C)$ and $^{14}C(^{3}H)$ push button channels which gave backgrounds of 14 counts/minute for ^{3}H and 10 counts/minute for ^{14}C.

Because chemical quenching varied between samples it was necessary to carry out corrections for quenching. The known methods for quench correction are the internal standard, the external standard and the channel ratio. For the double labelling techniques the external standard ratio method, (incorporated into the instrument), was used to calculate disintegrations per minute, (dpm), from the counts per minutes, (cpm). Standard Quench Correction

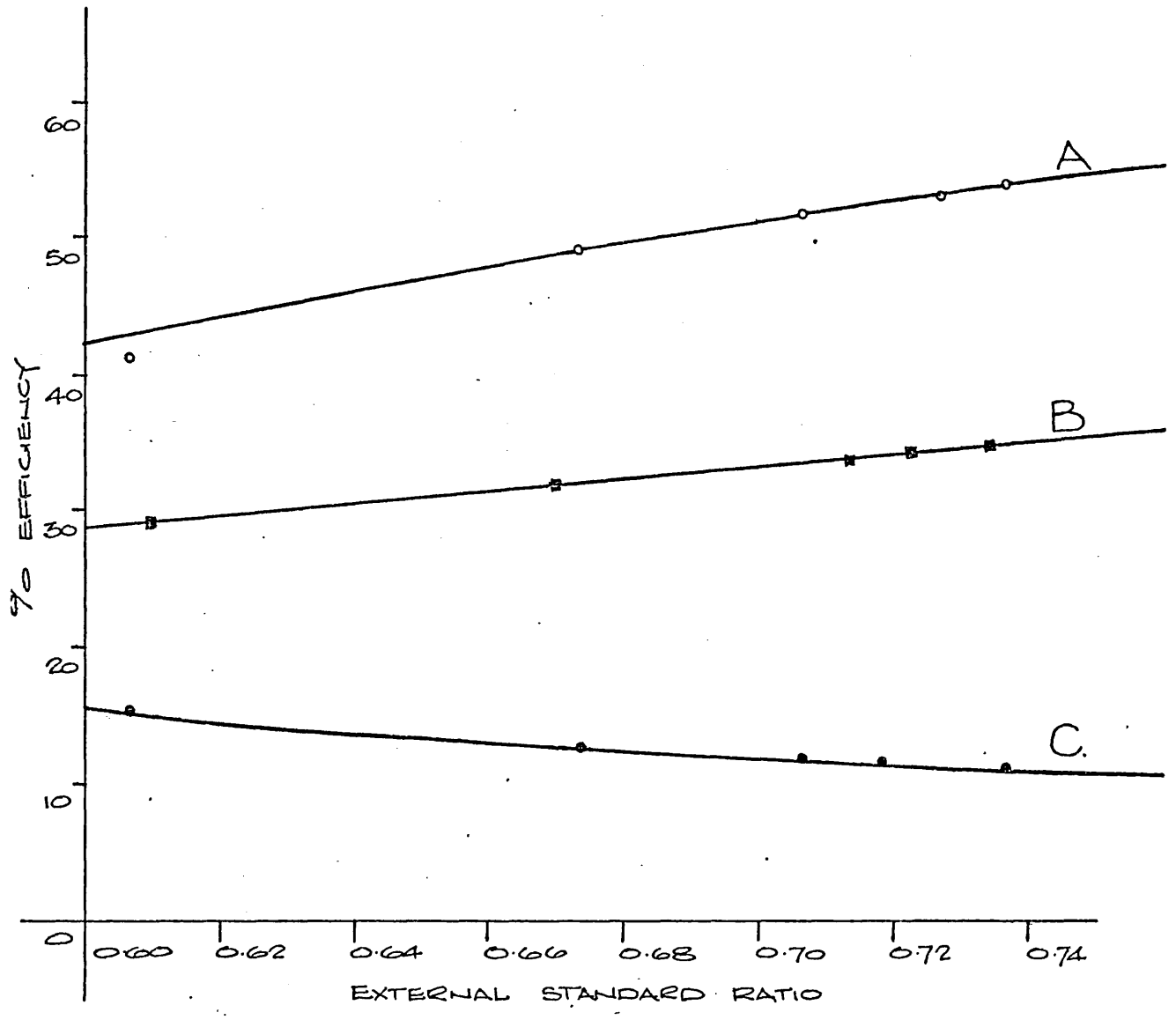


Figure 3 Quench Correction Curves.

A. ^{14}C counts in $^{14}C(^{3}H)$ channel.

B. ^{3}H counts in $^{3}H(^{14}C)$ channel.

C. ^{14}C counts in $^{3}H(^{14}C)$ channel.

Curves were obtained for ^{3}H and ^{14}C using nitromethane as the quenching agent, (Figure 3). The Triton X-100 phosphor used above is not very suitable for the counting of ^{3}H, [Turner, 1967]. The toluene blended mix of White, [1967], was consequently used for the counting of ^{3}H and ^{14}C, ie. 7g PPO, 600mg dimethyl POPOP, 150g of Naphthalene and 300ml of ethoxyethanol were dissolved in 1L of toluene. 100µl, (or less), of aqueous sample was added to 20ml of phosphor in a screw top glass vial. The vial was allowed to stand for 20 minutes to cool, and counts were recorded for 20 minutes or until the total counts exceeded 900,000.

PROTEIN ASSAY

a. Ultraviolet light adsorption measured at 280mµ on an Optica spectrophotometer was used as a rapid measurement of protein concentration.

b. More accurate measurements of proteins involved the method described by Lowry et al, [1951]. Alkaline copper sulphate solution was prepared by adding 1ml of 0.5% $CuSO_4 5H_2O$ in 1% sodium citrate to 50ml of 2% Na_2CO_3 in 0.1N NaOH. 4ml of this freshly prepared solution was allowed to stand for 10 minutes with 0.8ml of sample containing 10 - 100µg of protein. 0.4ml of diluted Folin-Ciocalteu phenol reagent, (1ml diluted to 2.3ml with water), was pipetted rapidly into the tube and thoroughly mixed. After standing for 1 hour the solution was read against a reagent blank at 750mµ using a red filter on the Optica Spectrophotometer. Protein concentration was calculated from a standard curve prepared using pure serum bovine albumin, (125µg/ml).

PROTEIN STAINS

Nigrosin

The dried cellulose acetate strip was floated on and then immersed into a solution of 0.001% Nigrosin in 2% acetic acid. The strip was left staining overnight and then quickly rinsed in distilled water and allowed to dry.

Naphthalene

A saturated solution of Naphthalene Black was prepared in 50:20:1 of

methanol : glycerol : acetic acid. The acrylamide flat gel was immersed in the filtered stain for 20 minutes with continual agitation. The gel was left overnight in 1% (v/v) acetic acid, and destaining was continued until the background became clear using a number of changes of 1% acetic acid.

Coomassie Brilliant Blue

The staining solution was prepared by dissolving 1.25g of Coomassie brilliant blue in a mixture of 454ml of 50% methanol and 46ml glacial acetic acid, and the insoluble material removed by filtering through Whatman's No. 1 filter paper. The acrylamide gels were placed in small tubes filled with the staining solution for 24 hours. The gels were then washed with distilled water and placed overnight in a destaining solution, (75ml acetic acid, 50ml methanol and 875ml water). The gels were stored in 7% acetic acid.

Benzidine Peroxide

Benzidine peroxide is a specific stain for haemoglobin and haemoglobin-haptoglobin complexes. Fresh stain was prepared by adding 30mg of ammonium chloride and 40µl of 30% H_2O_2 to 20ml of benzidine, (0.2% in 0.5% acetic acid). Gels were placed in a small volume of stain until blue bands were visible, normally after fifteen minutes.

PHOSPHOROUS ASSAY

The modified method of Fiske and Subba Row, [1925], as described by Bartlett, [1956], was used for the phosphorous assay.

2.0ml of sample and 0.5ml of 10 N H_2SO_4 were placed in a 12ml conical centrifuge tube and heated in a 150-160°C oven for 3 hours. 2 drops of 30% H_2O_2 were added and the tubes returned to the oven for one and a half hours. 4.4ml of water plus 0.2ml of 5% ammonium molybdate plus 0.2ml of Fiske-Subba Row reagent were added, mixed thoroughly and heated for 7 minutes in a boiling water bath, with marbles covering the tubes. The optical density at 830mµ was recorded using the Optica Spectrophotometer equipped with a red sensitive phototube. A phosphorous standard curve was prepared using a sodium phosphate salt solution containing 4.0mg of phosphorous.

(The Fiske-Subbarow reagent was prepared by adding 0.5g of 1-amino-2naphthol-4-sulphonic acid to 200ml of freshly prepared 15% anhydrous sodium bisulphite. The solution was stirred and 1.0g of anhydrous sodium sulphite added. The solution was filtered and stored in a dark bottle).

OTHER MATERIALS

Materials were generally of analar grade and obtained from The British Drug House Ltd.

Other materials:

Acrylamide (monomer) - stored at $4^{o}C$	-	Serva Heidelberg
Blue Dextran	-	Pharmacia Fine Chemicals
α chymotrypsinogen (bovine pancreas)	-	Sigma Chemicals
Coomassie Brillant Blue	-	"
Cytochrome C (horse heart)	-	Koch Light Ltd.
D-(^{14}C) glucose (sp. act. 16.7μCi/ml)	-	The Radiochemical Centre, Amersham
L-(^{14}C) glucose (" " 16.7μCi/ml)	-	"
D-(^{3}H) glucose (" " 2.8mCi/ml)	-	"
Methyl methacrylate polymers	-	Arthur D. Little Research Institute
Nigrosin	-	G. T. Gurr Ltd.
Phloretin	-	Fluka
PPO (scintillation grade)	-	Pachard Instrument Co.
POPOP (scintillation grade)	-	Koch Light Ltd.
dimethyl POPOP (scintillation grade)	-	"
Sephadex G-200 and G-75	-	Pharmacia Fine Chemicals
Serum albumin (bovine)	-	Koch Light Ltd.
Serum globulin (bovine)	-	"
Temed	-	Kodak Ltd.
Versapore filters	-	Millipore Ltd.

SECTION 3 RESULTS

MEMBRANE PROTEINS SOLUBILIZED BY BUTANOL EXTRACTION

THE COMPOSITION OF THE AQUEOUS PHASE

The aqueous layer obtained after the butanol extraction of water washed stroma was found to contain practically all the membrane protein and was essentially lipid-free. The composition of the aqueous phase, shown in Table 1, was in agreement with the results reported by other workers, [Maddy, 1966, and Rega et al, 1967]. The phosphorous determinations indicate the concentration of phospholipids, and from Table 1 it is clear that the ghosts contained approximately 3.0mg/ml of phospholipid.

TABLE 1 Composition of the prepared ghosts and aqueous phase. (The number of determinations is given in parenthesis)

Sample	Conc. of protein (mg/ml)	% recovery of protein	Conc. of phosphorous (mg/ml)	% recovery of phosphorous
White ghosts	3.061 (4)	100	103.0	100
Aqueous phase	1.699 (2)	83.3	5.094	7.4

DISTRIBUTION OF GLUCOSE AFTER BUTANOL EXTRACTION

As the aqueous phase contained over 80% of the protein and the butanol phase contained over 90% of the lipid, it was thought that some indication of the presence of a D-glucose binding component could be demonstrated if the extraction was carried out in the presence of glucose, and the distribution of glucose in the butanol and aqueous phase measured. An experiment was therefore carried out in which a comparison was made between the distribution of D-glucose, (A), and a control, (B), containing either an inhibitor of the hexose transport system or a sugar with a low affinity for the carrier. For (A) and (B) a series of 50ml polyethylene tubes were prepared as shown below:-

	A	B
Water washed stroma (containing approx. 3.5mg/ml protein)	26.25ml	26.25ml
Deionised distilled water	1.25ml	-
Mercuric chloride (2×10^{-4}M in water)	-	1.25ml
D-[^{14}C] glucose (containing 1.78mg/ml D-[^{12}C] glucose)	2.5ml	2.5ml

The final mercury concentration of 10^{-5}M used in B was sufficient to inhibit the transport of glucose across red blood cells, [LeFevre, 1948]. Sen and Widdas, [1962], showed that the half saturation concentration for D-glucose at room temperature was approximately 2. Hence the 4mM concentration of glucose used above should saturate the carrier system to about 70% for this and subsequent experiments carried out at room temperature.

After butanol extraction, 100µl samples were taken from the aqueous and butanol phases for G-M counting, and the results are given in Table 2. Rega et al, [1967], reported that they found a thick white emulsion at the interface after the first centrifugation and a thin insoluble film after the second centrifugation. In these experiments a 'straw coloured' thin insoluble film was observed in only about 60% of the experiments used to prepare the solubilized protein loaded with glucose. Although variations in the experimental conditions were made it was not possible to determine a reason for the presence of a film in some experiments but not in others. When the film was present, it was solubilized, and 100µl samples were taken for G-M counting, (Table 2). The interface film could be partially solubilized using either NaI, (20%), or Triton X-100, (10%), but complete solubilization was obtained using 1ml of phenol : acetic acid : water, (1:1:1, w/v/v), as described by Bagdasarian et al, [1964]. However, by using this solvent the efficiency of the counting was lowered due to the presence of phenol.

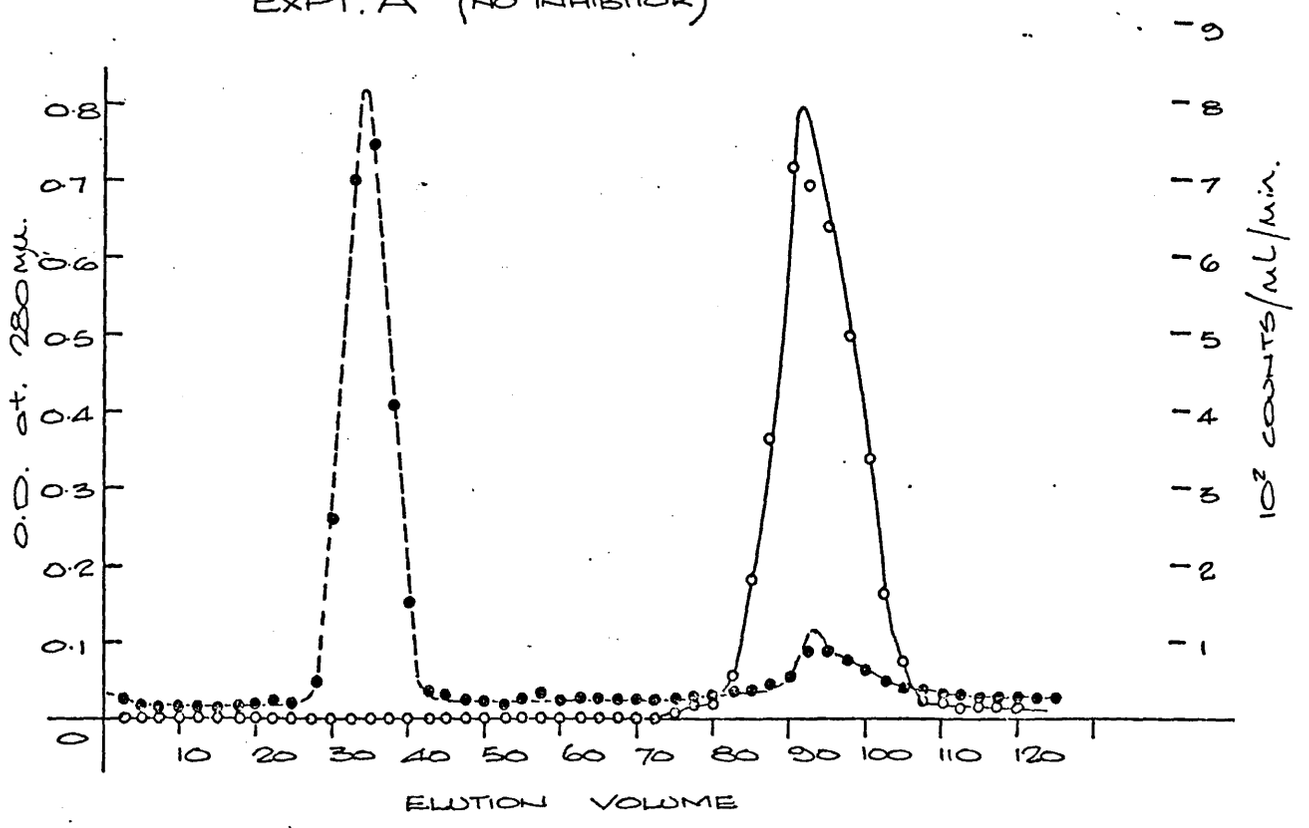
TABLE 2 Distribution of glucose after butanol extraction

Sample	Number of experiments	A (inhibitor none) cpm/ml	B (inhibitor $HgCl_2$) cpm/ml	Ratio (A)cpm/(B)cpm
Emulsion	7	47,950	47,800	1.00
Aqueous phase	7	35,420	35,380	1.00
Butanol phase	7	785	1,050	0.75
Interface in PhOH:AcOH:H_2O	4	26	25	1.04

As L-glucose has a very low affinity for the carrier a similar experiment was carried out to compare the distribution of D-glucose and L-glucose. In this experiment the two series of tubes were prepared with A containing 2mM D-(^{14}C) glucose and B containing 2mM L-(^{14}C) glucose. During different stages of the butanol extraction, (Table 3), 100µl samples from the aqueous and butanol phases were added to 10ml of Triton X-100 phosphor for Liquid Scintillation Counting.

TABLE 3 Distribution of glucose during butanol extraction

Stage of extraction	Sample	A(D-glucose) cpm/ml	B(L-glucose) cpm/ml	Ratio (A)cpm/(B)cpm
	Emulsion	17,610	17,730	0.99
First centrifugation	Aqueous phase	24,620	24,820	0.99
"	Butanol phase	761	795	0.96
Second centrifugation	Aqueous phase	5,690	5,730	0.99
"	Butanol phase	181	206	0.88



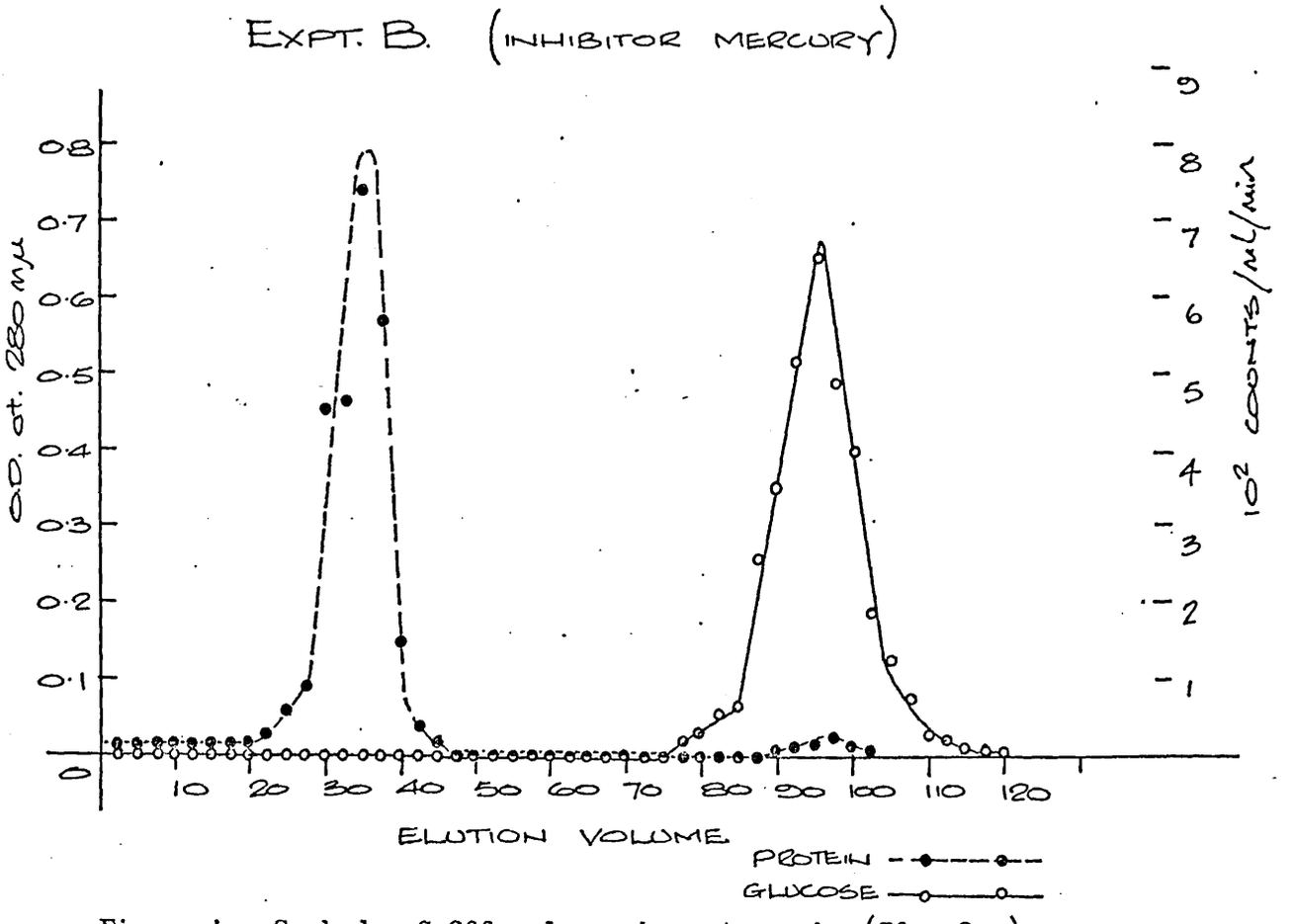


Figure 4 Sephadex G-200 column chromatography (30 x 2cm).
Sample: 10mg of protein incubated with D-glucose.
Buffer: 150mM NaCl, 10mM Na_2HPO_4-KH_2PO_4 (pH 7.4)

The experiments indicated that butanol was a poor solvent for glucose, but in both Table 2 and Table 3 the D-glucose counts per minute, (cpm), in the test experiments were lower than in the two controls. Assuming that the control experiments demonstrated the passive distribution of glucose in the two phase system, the D-glucose counts lost from the butanol phase in the test experiments were probably in the interface or aqueous phase. Too few radioactive counts were found associated with the interface for any conclusions to be made, and the ratio of (A)cpm/(B)cpm for the aqueous phase and emulsion was the same in both experiments. However, as the aqueous phase contained the bulk of the glucose, a small increase of D-glucose might have been difficult to observe, and the experiments were too imprecise for conclusive interpretations to be made.

GEL CHROMATOGRAPHY

The aqueous phases from A and B in the first experiment, (Table 2), were used for gel chromatography. Butanol was removed from the aqueous phase by evaporation and the solution was concentrated until it contained approximately 10mg of protein/ml. 1ml of the protein solution was then placed on a standardised Sephadex G-200 column and eluted with phosphate buffer. 2.5ml fractions were collected at a flow rate of 7.5ml/hour and 100µl samples were taken from each fraction for G-M counting. Protein concentration was then estimated by measuring each sample at 280mµ. Figure 4 illustrates the typical elution patterns which were obtained.

The protein was separated on Sephadex G-200 into high and low molecular weight fractions. 90% of the protein was eluted in the 'zone volume' of the column, (cf. Figure 1), indicating the presence of a protein with a molecular weight of over 800,000. Rega et al, [1967], reported that the low molecular weight material was not protein but was probably composed of oxidised lipids.

The elution patterns showed that practically all the glucose was eluted as free glucose, and that the elution of glucose appeared to be unaffected by the

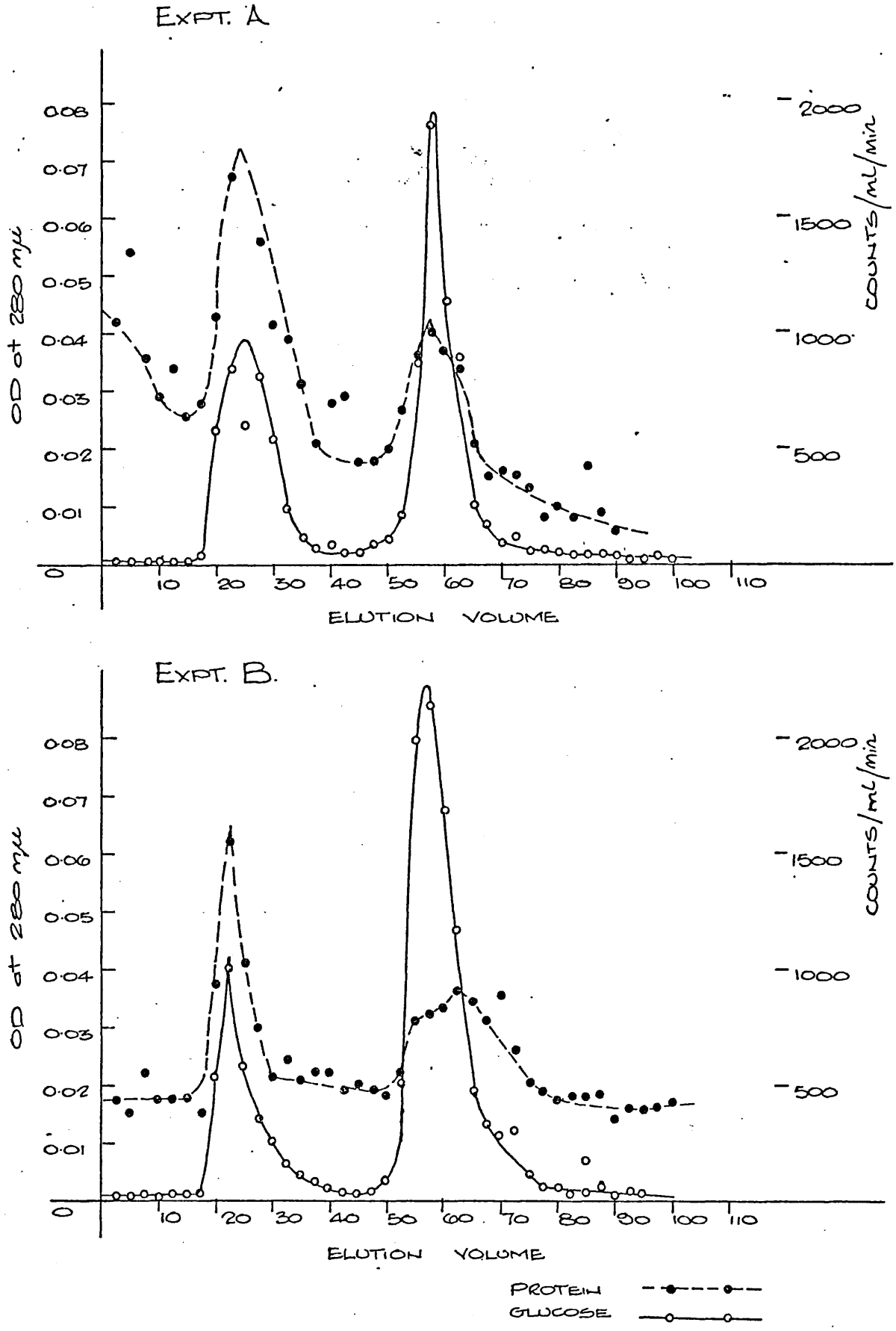


Figure 5 Sephadex G-75 column chromatography (20 x 2cm).
Sample: Fractions taken from Sephadex G-200 column.
Buffer: 150mM NaCl, 10mM Na_2HPO_4-KH_2PO_4 (pH 7.4).
Flow rate: 7.5ml/hour.

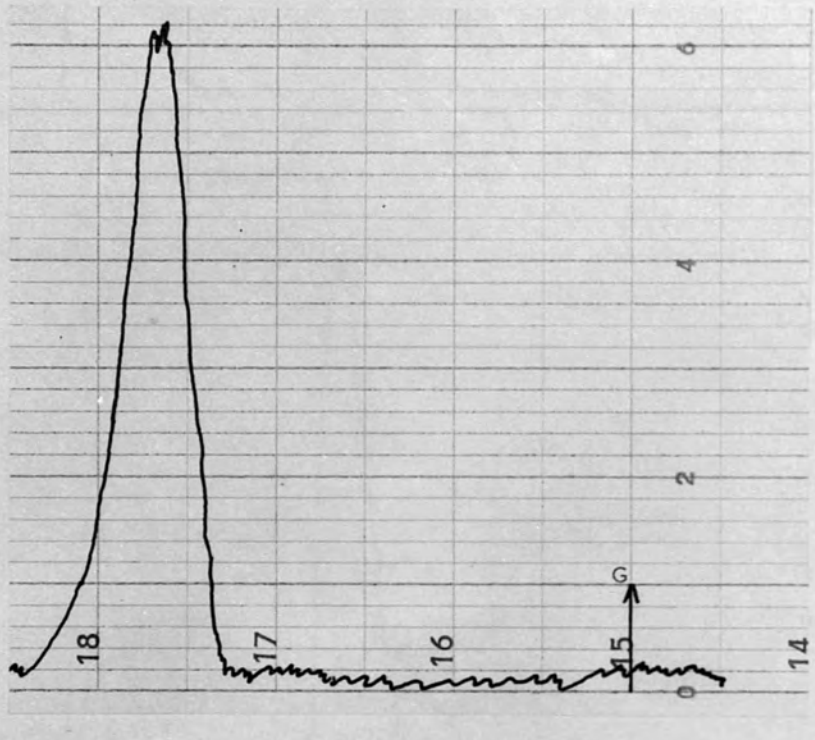


Figure 6 Cellulose Acetate Membrane Electrophoresis in Barbitone buffer, (pH 8.6), for two hours at 1mA/strip.

Top: CAM of sample blue dextran and D-(^{14}C) glucose.

Bottom: Radioactive scan of CAM.

presence of the protein. A negligible amount of glucose, (35 - 50 cpm/ml), was eluted with the major protein peak in the zone volume. As this occurred for both A and B, it must have been due to a non-specific association between the protein and the glucose.

Gel chromatography, with a smaller fractionation range, had to be used to separate the free glucose from the low molecular weight material which had been eluted together in the same fractions. The fractions which were eluted between the volumes 75 - 105ml on the Sephadex G-200 column were pooled and concentrated by ultrafiltration. 2ml of the concentrated solution was placed on a standardised Sephadex G-75 column and eluted with phosphate buffer. Radioactive counts and protein estimations were carried out as with the Sephadex G-200, (Figure 5).

Two absorption peaks were observed at 280mμ for the low molecular weight material. D-glucose appeared to be associated non-specifically with the first peak, which was illustrated in both A and B. To obtain further information about this material, thin layer chromatography was carried out but gave no satisfactory results.

ELECTROPHORESIS

Cellulose Acetate Membrane (CAM)

The movement of protein, glucose and a combination of the two were compared using Cellulose Acetate Membrane Electrophoresis. It was necessary at first to estimate the electro-osmotic effect during electrophoresis, moving glucose towards the cathode, using a mixture of Blue Dextran and radioactively labelled glucose. 5μl of Blue Dextran together with D-(^{14}C) glucose, [and D-(^{12}C) glucose, 1.78mg/ml], were placed on an impregnated CAM 1cm from the cathode. Electrophoresis was carried out at 1mA using Barbitone buffer, (pH 8.6), until the Blue Dextran had travelled approximately two thirds of the distance towards the cathode. After electrophoresis, which took about two hours, the strip was allowed to dry and was then scanned for radioactivity, (Figure 6). Both

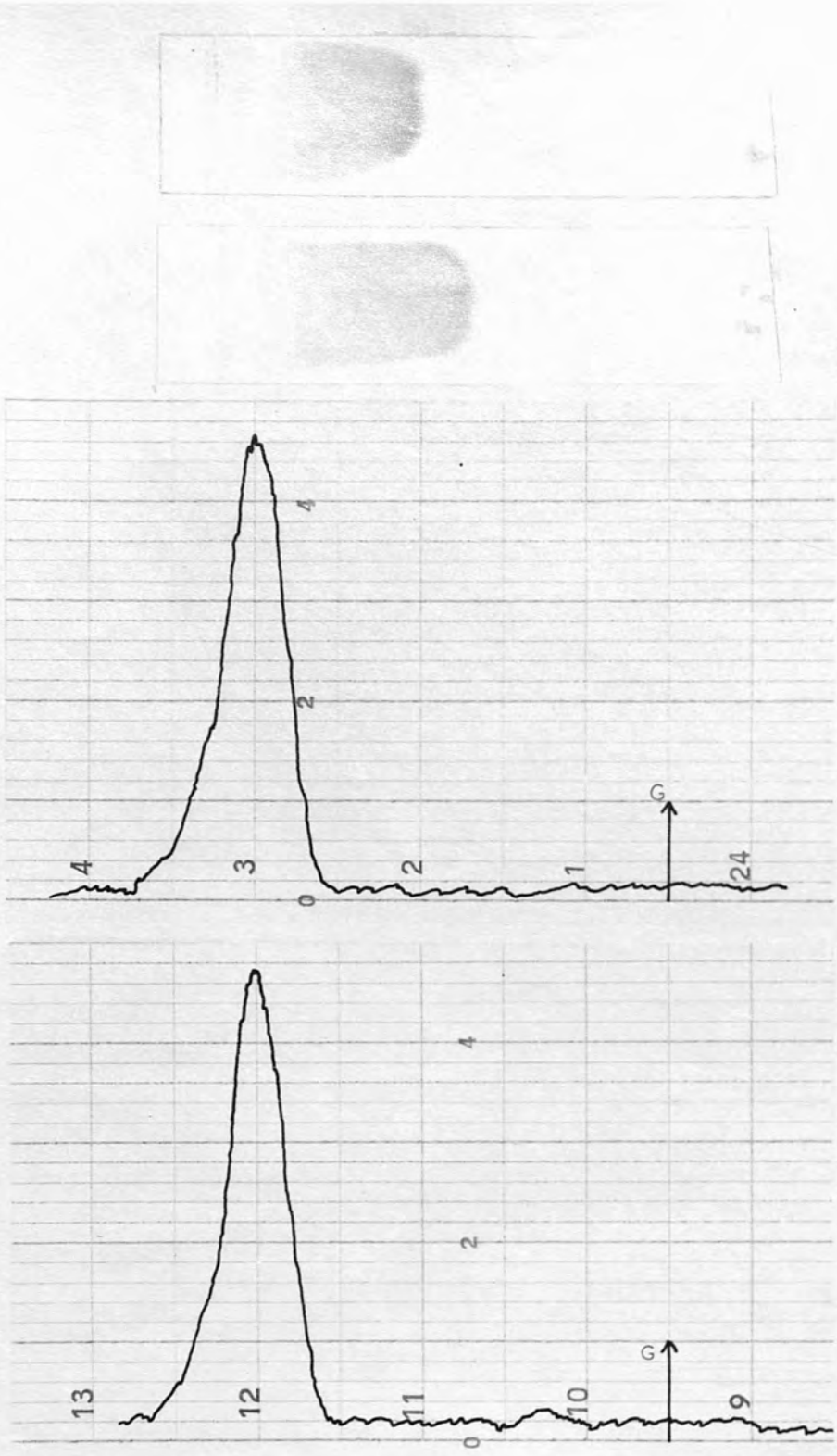


Figure 7 Cellulose Acetate Membrane Electrophoresis in Barbitone buffer, (pH 8.6), for one and three quarter hours, 1mA/strip.

Top to Bottom:

1. CAM of sample protein and D-(^{14}C) glucose.
2. CAM of sample protein alone.
3. Rad. scan of CAM sample protein and D-(^{14}C) glucose.
4. Rad. scan of CAM sample D-(^{14}C) glucose alone.

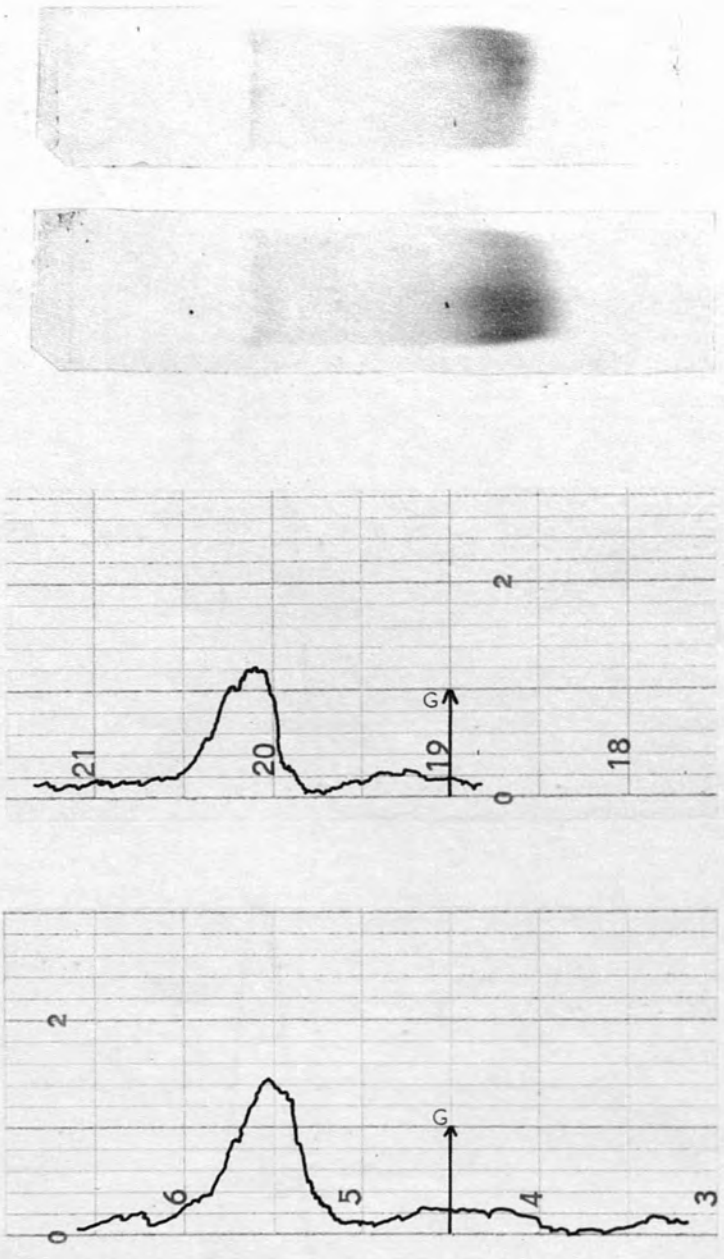


Figure 8 Cellogel CAM Electrophoresis in Barbitone buffer, (pH 8.6), for two hours at $1\frac{1}{2}$mA/strip.

Top to Bottom:

1. CAM of sample protein and D-(^{14}C) glucose.
2. CAM of sample protein alone.
3. Rad. scan of CAM sample protein and D-(^{14}C) glucose.
4. Rad. scan of CAM sample D-(^{14}C) glucose alone.

Blue Dextran and glucose were found to have moved by electro-osmosis 5.2cm and 6.6cm respectively. Having estimated the electro-osmotic effect, the positions for glucose and protein could be marked so that the two would cross each other during electrophoresis.

For each experiment two control membranes with protein and glucose alone were run simultaneously against the test membrane. On the test membrane, 5µl of labelled D-glucose were placed 2cm from the anode. Protein and glucose were placed on the control membranes in similar positions. Electrophoresis was carried out for $1\frac{3}{4}$ hours at 3mA using Barbitone buffer, (pH 8.6). The membranes were then allowed to dry, scanned for glucose and stained for protein, (Figure 7).

These experiments were repeated using cellogel membranes instead of CAM, but glucose was placed in the centre of the strip, (Figure 8). The results of five experiments using CAM and the two using cellogel are summarised in Table 4.

TABLE 4 Cellulose Acetate Membrane Electrophoresis to compare the movement of protein and glucose
(Figures are mean with range in parenthesis)

Samples	Protein migration (cm)	% protein retardation	Glucose movement (cm)	% glucose acceleration
CAM				
Protein + glucose	3.25 (2.2-3.9)	10.1 (7.0-29.0)	6.50 (6.1-6.8)	3.1 (2.0-4.6)
Protein	3.65 (3.3-4.2)			
Glucose			6.30 (6.0-6.5)	
CELLOGEL				
Protein + glucose	4.1 (4.05-4.15)	8.8 (7.6-10.0)	2.8 (2.8)	7.6
Protein	4.5 (4.5)			
Glucose			2.6 (2.6)	

During CAM electrophoresis the protein solubilized by butanol extraction was not separated into any subunits. However, the migration of a single component was observed, followed by 'streaking' which was probably due to the presence of lipids. Resolution and reproducibility was greatly improved using the cellogel membranes. It was found that in all the experiments protein migration was retarded by the glucose, but the degree of redardation varied greatly between experiments. When using CAM it was difficult to show whether the retardation of protein by glucose was due to a physical association or chemical binding. Hence the experiments were repeated using gel electrophoresis to demonstrate whether specific protein components were bound with or retarded by glucose.

Another effect observed using CAM electrophoresis was the increase in electro-osmosis of glucose during the migration of protein towards the anode. As the binding of glucose and protein would have been expected to reduce the movement of glucose, the observed increase of the glucose movement was difficult to reconcile with the simultaneous retardation of protein migration.

Acrylamide Flat Gel

To obtain a more reproducible protein migration pattern, acrylamide flat gel electrophoresis was used in place of CAM. As gel electrophoresis separates membrane proteins into their subunits it was hoped that a particular component in the protein extract might be influenced by or might influence the zone of glucose. The flat gel contained four sample slots to allow protein and glucose controls to run simultaneously against the test samples.

5% polymerised acrylamide gels, containing the required sample slots, were dialysed against 0.1M Barbitone buffer, (pH 8.6), and then placed in the electrophoretic tank. The aqueous phase was reduced by evaporation to give a protein concentration of approximately 30mg/ml. 10µl aliquots of concentrated protein solution were placed in the slots nearest to the cathode and it was necessary to check that the second sample would not influence migration whether it contained either radioactively labelled D-(^{14}C) glucose

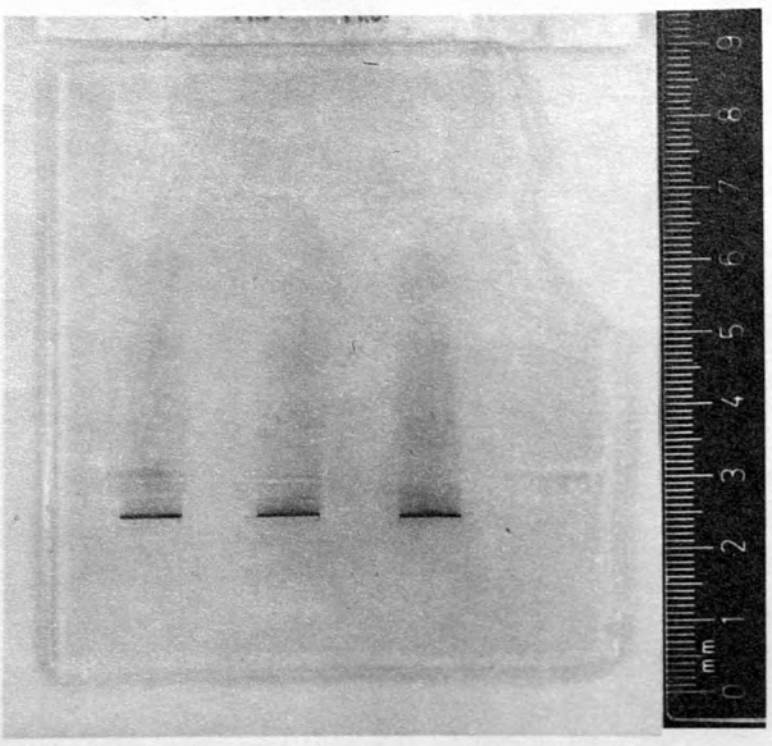


Figure 9 Acrylamide Flat Gel Electrophoresis in Barbitone buffer (pH 8.6), for five hours at 130 volts.

Left to Right:

1. Sample of protein and buffer.
2. Sample of protein and D-(^{14}C) glucose.
3. Sample of protein alone.
4. Sample of D-(^{14}C) glucose alone.

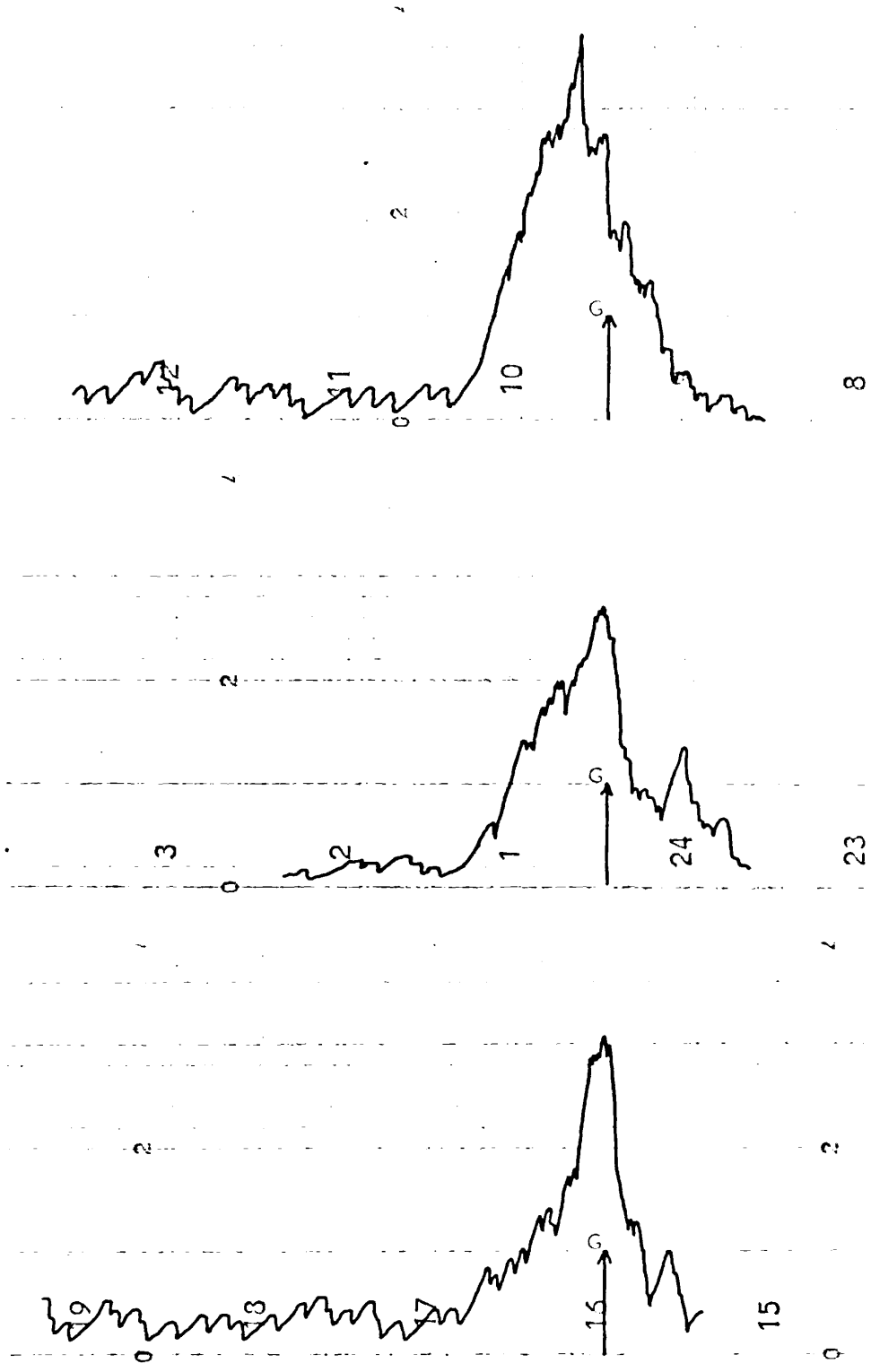


Figure 10 Radioactive Scans of Acrylamide Flat Gel in Barbitone buffer.

Samples Top: Protein and D-(^{14}C) glucose.

Centre: Protein and L-(^{14}C) glucose.

Bottom: D-(^{14}C) glucose alone.

or buffer, (Figure 9). Care was taken to ensure that the slots were filled completely without overflowing. Radioactively labelled glucose was run alone as a control experiment to demonstrate any electro-osmotic effect. Electrophoresis was carried out at 130v for five hours and the gel was then removed, scanned for radioactivity and stained for protein with Naphthalene Black. Figure 9 illustrates a major problem due to the apparent association of a large proportion of the protein which was unable to penetrate the gel. Certain experiments had to be discarded because little or no protein appeared to enter the gel. The protein that had entered the gel was resolved into three bands which had migrated approximately 1.25cm, 2.3cm and 3.2cm, (Figure 9). Neither the presence of glucose nor buffer in the second sample slot appeared to influence the migration of these protein bands.

Scanning of those samples containing radioactively labelled glucose alone showed that glucose did not move in either direction, and that the electro-osmotic effect observed with CAM electrophoresis was negligible, (Figure 10). As protein migrated through a zone of glucose, the glucose tended to move a short distance towards the anode, (ie. in the direction of the protein). This apparent association of glucose and protein could be demonstrated with either D-glucose or L-glucose, (Figure 10). Although the exact measurement of glucose movement was not possible by radioactive scanning, the mean values obtained were 0.30cm, (range 0.15 - 0.50), for D-glucose and 0.15cm, (range 0.10 - 0.25), for L-glucose. The association between glucose and a particular protein component could not be detected.

To reduce the amount of protein association occurring in the sample slot, a 'discontinuous' buffer system was used. Two gels were prepared, one using Tris-Citrate buffer, (0.075M Tris and 0.0025M Citric Acid, pH 8.6), and the other using Tris-Citrate buffer, (pH 8.6). plus 6M Urea. 10µl samples of 3% protein, radioactively labelled D-(^{14}C) glucose and L-(^{14}C) glucose were placed in their respective slots. The electrode compartments contained 0.5M Borate buffer, (pH 8.6). Electrophoresis was carried out at 500v for

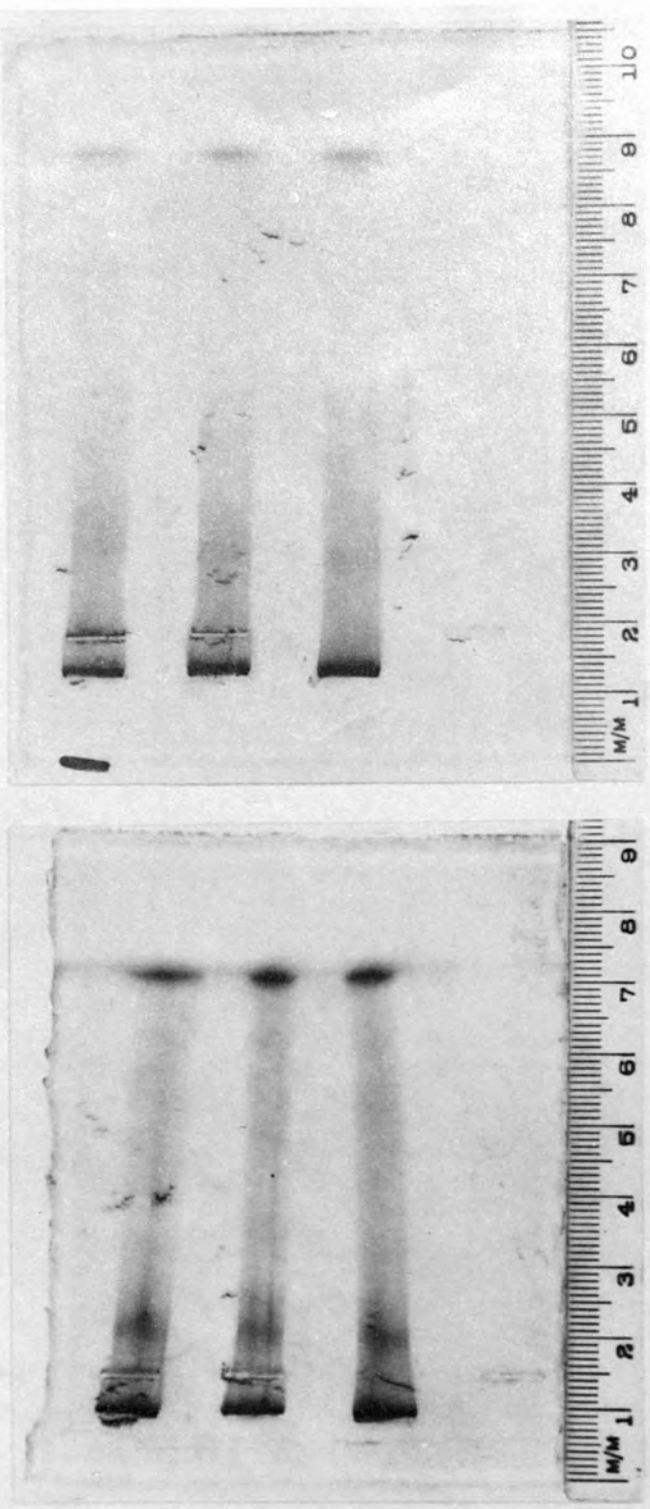


Figure 11 Acrylamide Flat Gel Electrophoresis at 500 volts for three hours in buffer,

Top: Tris-Citrate/Borate, (pH 8.6)
Bottom: Tris-Citrate:Urea/Borate, (pH 8.6)

Left to Right:

1. Sample of protein and L-(^{14}C) glucose.
2. Sample of protein and D-(^{14}C) glucose.
3. Sample of protein alone.
4. Sample of D-(^{14}C) glucose alone.

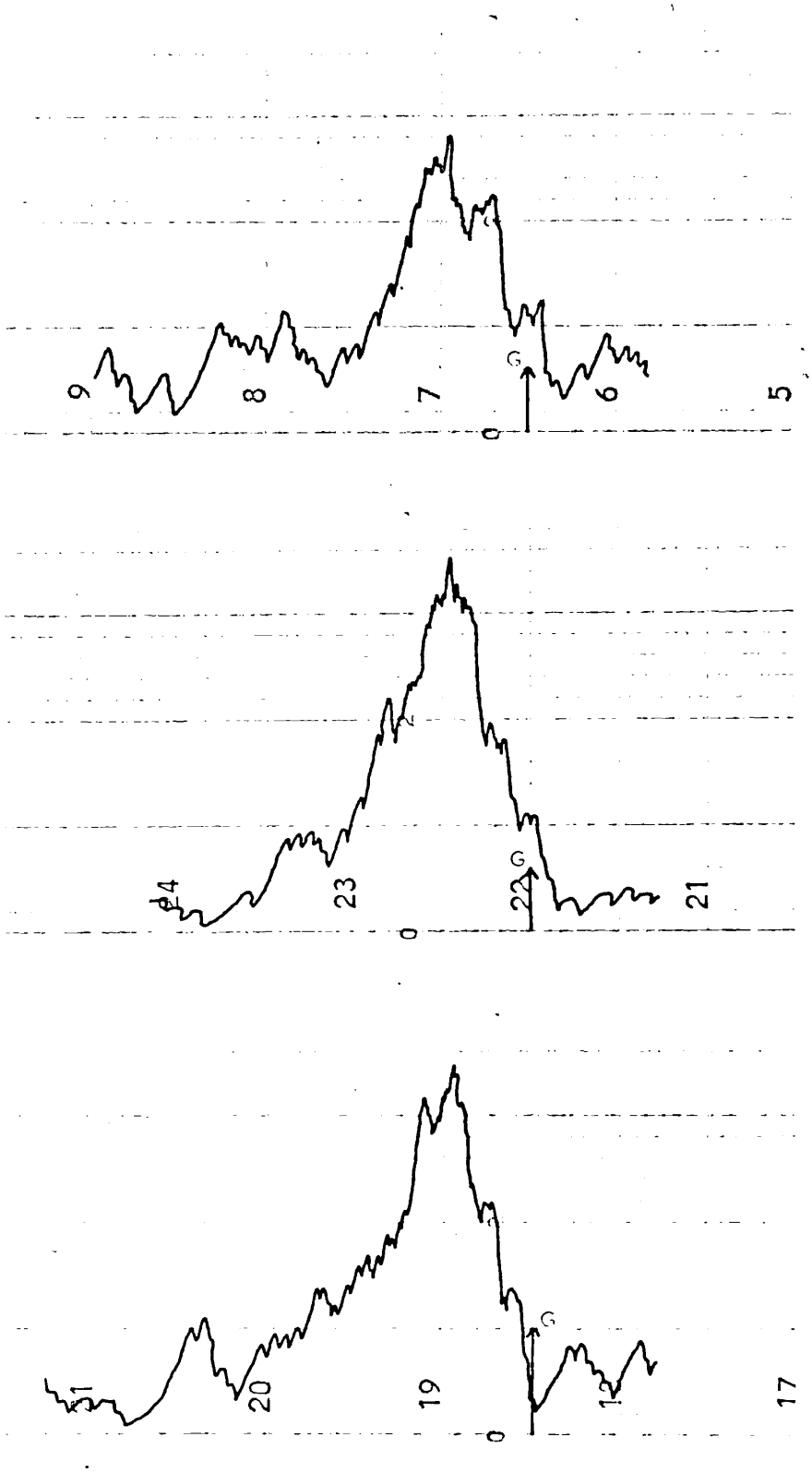


Figure 12 Radioactive Scans from Acrylamide Flat Gel in Tris-Citrate/ Borate buffer.

Samples Top: Protein and L-(^{14}C) glucose.

Centre: Protein and D-(^{14}C) glucose.

Bottom: D-(^{14}C) glucose alone.

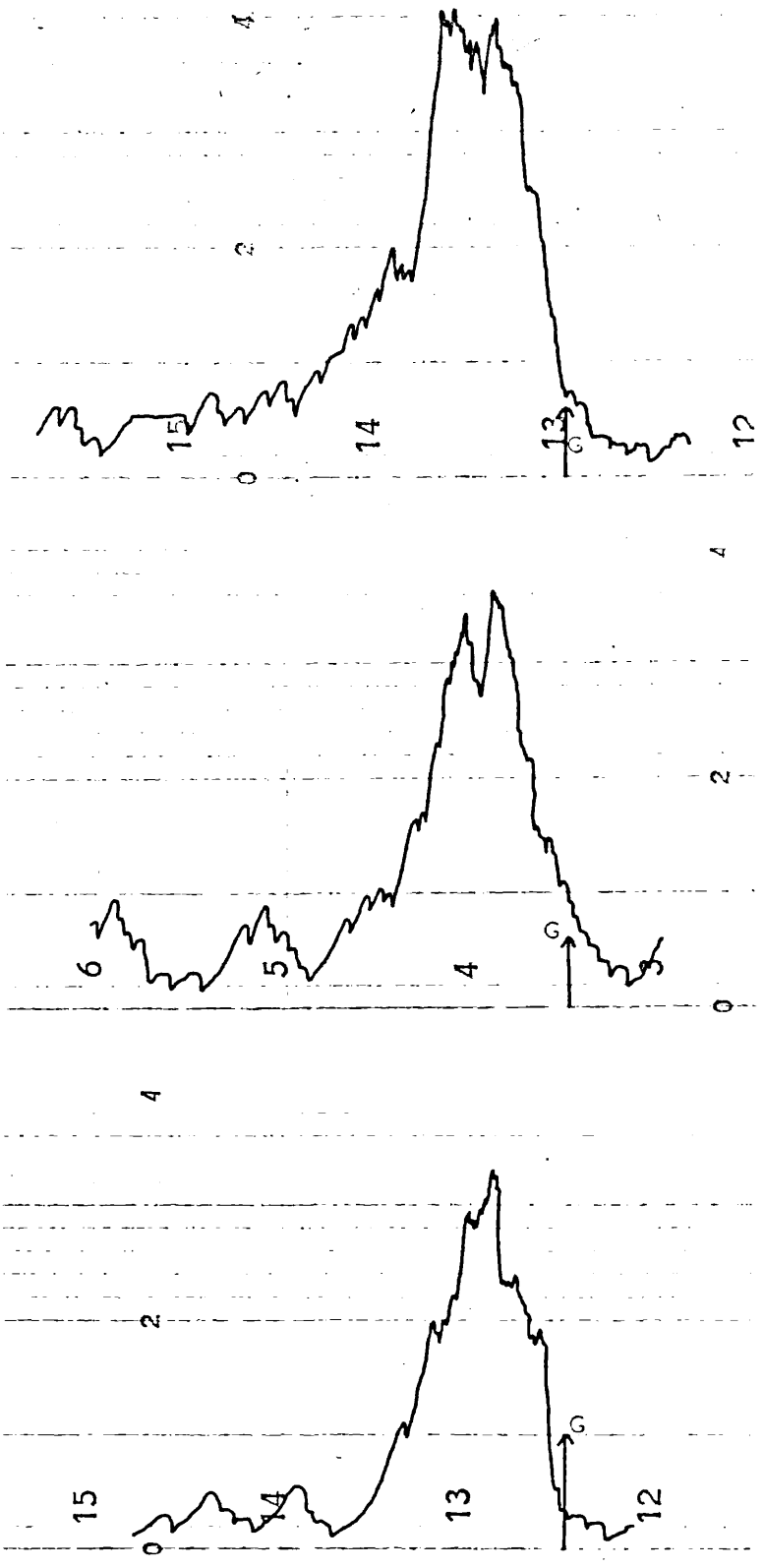


Figure 13 Radioactive Scans from Acrylamide Flat Gel in Tris-Citrate: Urea/Borate buffer.

Samples Top: Protein and L-(^{14}C) glucose.

Centre: Protein and D-(^{14}C) glucose.

Bottom: D-(^{14}C) glucose alone.

three hours when the brown borate line had reached 3cm from the anode end of the gel. The amperage across the gel fell from 14mA to 6mA during the first 30 minutes. After electrophoresis the gels were scanned for radioactivity and then stained for protein with Naphthalene Black.

The typical results from the two gels can be seen in Figures 11, 12, and 13. The discontinuous buffer system appeared to improve the migration of the protein subunits. Although considerable 'streaking' of the protein occurred, a number of bands were observed, especially when urea was added to the buffer system. As before, the migration of the proteins was unaffected by the presence of glucose in the second sample slot, (Figure 11).

In the Tris-Citrate/Borate discontinuous system, free glucose moved approximately 1.0cm towards the anode. This was probably caused by the presence of borate, as sugar reacts with borate to form charged sugar-borate ions. This movement of glucose was increased by about 0.2cm in the experiment where protein migration occurred. This increase was observed by both D-glucose and L-glucose, (Figure 12). The inclusion of urea in the buffer increased the amount of protein penetrating the gel, which appeared to form a second peak of glucose in addition to the free glucose peak about 0.5cm apart. This was observed for both D-glucose and L-glucose.

Acrylamide flat gel electrophoresis demonstrated a non-specific movement of glucose when the proteins solubilized by butanol extraction migrated through a glucose zone. This association was also found when urea, which denatures protein, was included in the gel buffer.

DIALYSIS EXPERIMENTS

The distribution of small ions and molecules between the internal and external fluids during dialysis of a protein solution is dependent on the osmotic effect of the protein. If any of the molecules present were to bind to the protein, the distribution of these molecules would be altered. The degree of binding to protein can therefore be estimated by comparing

TABLE 5 D-Glucose binding shown by dialysis

Sample	Age of prepn. (days)	D-Glucose cpm/ml				L-Glucose cpm/ml			
		internal		external		internal		external	
		0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr
Stroma	2	7,780	9,100	8,900	8,260	8,660	8,720	8,640	8,940
Stroma	12	6,780	13,400	6,540	6,220	8,240	8,020	8,760	9,320
Solubilized protein	5	7,760	8,240	8,040	7,280	8,540	8,200	8,660	8,960
Solubilized protein	15	8,560	42,400	8,240	6,540	8,600	9,300	8,620	8,760
Solubilized protein (filtered through a millipore)	18	6,370	6,350	6,790	6,660	8,950	7,550	7,790	7,270

TABLE 6 Ratios calculated from Table 5

Sample	Age of prepn. (days)	Ratio of Internal (cpm/ml)/External (cpm/ml)			
		D-Glucose		L-Glucose	
		0hr	24hr	0hr	24hr
Stroma	2	0.87	1.10	1.00	0.98
Stroma	12	1.04	2.15	0.94	0.86
Solubilized protein	5	0.97	1.13	0.99	0.92
Solubilized protein	15	1.04	6.48	1.00	1.06
Solubilized protein (filtered through a millipore)	18	0.94	0.95	1.15	1.04

identical experiments with a control, in which the binding component had been replaced by molecules known not to bind protein. Simple dialysis experiments were conducted to estimate the degree of binding of D-glucose to protein solubilized by butanol extraction or water washed stroma relative to the binding of L-glucose in identical experiments.

Dialysis experiments using D-glucose and L-glucose were carried out simultaneously. 10ml of a 10mM glucose solution, (containing radioactively labelled ^{14}C glucose), was added to 90ml of 10mM NaCl, 5mM Phosphate buffer, (pH 7.4), in a stoppered conical flask. 0.5ml of the same stock glucose solution was added to 4.5ml of buffered, water washed stroma or protein solubilized by butanol extraction. 50µl samples were taken from both solutions for Liquid Scintillation Counting. The protein solution was placed in a previously washed Visking tube bag and dialysis carried out as described in the methods section. As far as possible the speed of rotation of the magnetic stirrers was kept the same for both experiments. Dialysis was carried out overnight, as time course experiments had shown that ~~that~~ this produced a steady state. 50µl samples were then taken from the external buffer solution and the internal solution for radioactive analysis in the Liquid Scintillation Counter.

These experiments clearly indicated that some specific binding of D-glucose by the protein had occurred in the internal solution. However, reproducible results for the degree of binding were not obtained, and it appeared that the binding was influenced by the age of the stroma or protein. Experiments were therefore carried out using fresh samples and then repeated on the same refrigerated sample ten days later. From the results, (Table 5), and the calculated ratios, (Table 6), it was evident that the counts for D-glucose increased in the internal fluid and decreased in the external buffer for both stroma and protein solubilized by butanol extraction. However, in the experiments using L-glucose there was no comparable change in glucose counts in the internal fluid although the external fluid showed a small increase in

counts. Assuming that L-glucose acted as a control, these results indicated a binding of D-glucose to the stroma or protein within the Visking tubing.

These results also showed conclusively that the degree of binding was affected by the age of the sample. The increase of D-glucose bound after leaving the samples for a number of days suggested that these samples were probably contaminated.

The experiment using protein solubilized by butanol extraction was therefore repeated with sterilised apparatus and buffers. Also before loading the protein sample with glucose, any bacteria present were removed from the solution by filtering through a millipore filter of pore size 0.45μ.

The results obtained for these filtered samples, (Tables 5 and 6), indicated that the previous binding of D-glucose had been eliminated. This elimination of binding was assumed to be due to the removal of bacteria, although the millipore filtering could possibly cause some denaturation of the protein and any carrier components.

In confirmation of bacterial contamination it was found that both the stroma and solubilized proteins readily formed colonies on agar plates, and microscopic studies indicated that short rod bacteria were present. For all future experiments the sterilisation precautions, mentioned in the methods section, were carried out.

It was necessary, however, to discard dialysis as a method to demonstrate the binding of D-glucose because any positive results obtained would be unreliable for a method of such long duration and in which the maintenance of sterility could not be assured. Although working at a low temperature of $4^{o}C$ would reduce the effects of chance contamination, the continual stirring necessary during dialysis and the time course, involved too great a hazard.

INTERFACIAL MATERIAL

MILLIPORE EXPERIMENTS

Millipore experiments were carried out to study the interfacial material found after centrifugation of the water washed stroma-n-butanol emulsion. When a sample of the emulsion, formed by shaking water washed red cell stroma with n-butanol, was filtered through a hydrophobic (Versapore) filter, the emulsion was partially separated into a clear butanol phase and a cloudy aqueous phase. After the filter had been rapidly washed with acetone and allowed to dry, a 'straw' coloured material was observed on the surface. This material resembled the thin insoluble interface observed after the second centrifugation during the extraction of protein using n-butanol. If the stroma was loaded with D-(^{3}H) glucose and L-(^{14}C) glucose, prior to the addition of butanol and filtering, a retention of D-glucose was observed on the filter in preference to L-glucose.

As the half saturation concentration is less than 0.5mM at 0°C, (Sen and Widdas, 1962), the stroma was loaded with a concentration of 1mM for both D-glucose and L-glucose. Radioactive D-(^{3}H) glucose and L-(^{14}C) glucose was added to the water washed stroma to obtain a ^{3}Hdpm/^{14}Cdpm ratio of five, [which reduced the ^{14}C counts in the ^{3}H(^{14}C) channel]. D-glucose and L-glucose were carefully weighed and then added to the stroma to give the final required concentration. Care was taken throughout the experiment to ensure that the temperature was kept between 0-4°C. n-Butanol was then shaken with this stroma as described in the methods section, and 1ml samples of the emulsion were passed through a Versapore filter. The mean results for the calculated disintegrations per minute, (dpm), of seven typical experiments are shown in Table 7. The calculation of the bound counts has been described in the methods section.

TABLE 7 The distribution of radioactive counts after filtration of butanol -water washed stroma emulsion

Sample	^{3}H dpm/ml	^{14}C dpm/ml	Ratio ^{3}H dpm/^{14}C dpm	Dpm bound
Emulsion	1,780,000	372,000	4.77	-
Filtrate	1,900,000	399,000	4.77	-
Acetone washing	190,000	41,300	4.60	-
Versapore filter	8,680	1,770	4.91	23%

It was found that the filtrate gave a similar ratio to that obtained for the emulsion. However, the acetone fraction showed a reduced content of ^{3}H dpm which varied from one experiment to another. The higher ratio of ^{3}H dpm/^{14}C dpm obtained with a Versapore filter indicated a retention of D-glucose on the filter.

The Effect of Inhibitors on the Retention of D-Glucose

Experiments were continued to investigate how the observed binding on the Versapore filter was affected by inhibitors which reduce the transport of glucose across the red cell membrane, (Table 8).

It was necessary to allow the stroma to equilibrate with a large volume of the competitive inhibitor, phloretin, because phloretin is taken up by both the carrier molecules and the lipids of the red cell membrane. Two experiments were conducted in sterilised 50ml polyethylene tubes. 0.6ml of absolute alcohol was added to 45ml of distilled deionised water in one tube, and 0.6ml of phloretin, [dissolved in absolute alcohol (10mg/ml)], was added to 45ml of distilled deionised water in the second. 5ml of water washed stroma was added to both tubes, which were then allowed to stand in an ice bucket for 10 minutes. The tubes were centrifuged at 14,500g for 15 minutes and the supernatant was decanted. The final concentration of phloretin in

TABLE 8 The Effect of Inhibitors on the Retention of D-Glucose by Versapore Filters

Inhibitor	Ratio of ^{3}H dpm / ^{14}C dpm		Dpm bound on filter	10^{-8} g D-glucose bound by 1ml of stroma
	Reaction mixture	Millipore		
1. None	5.06	4.92	-249	-0.17
2. "	5.04	4.74	-817	-0.56
3. Phloretin (0.4mM)	4.99	4.83	-913	-0.66
4. "	4.89	4.51	-102	-0.07
1. None	5.32	5.38	+554	+0.30
2. "	5.32	5.52	+119	+0.10
3. Mannose (50mM)	5.33	5.38	+150	+0.08
4. "	5.34	5.50	+198	+0.11
1. None	4.82	4.90	+135	+0.10
2. "	4.82	4.93	+218	+0.17
3. Mannose (50mM)	4.86	4.90	+ 63	+0.05
4. "	4.86	4.78	-176	-0.13
1. None	4.64	4.84	+375	+0.22
2. "	4.64	4.96	+519	+0.31
3. 3-0 methyl glucose (50mM)	4.98	5.10	+223	+0.16
4. "	4.98	4.94	- 73	-0.05

the inhibited experiment was 4×10^{-4}M. 4ml of stroma from both experiments were then taken for the millipore experiment. Neither the uninhibited nor the inhibited experiment showed a retention of D-glucose on the Versapore filter, (Table 8), although the interface material was still observed as a brown discolouration in the centre of the filters. The addition of absolute alcohol probably prevented any retention of D-glucose on the filter.

The stroma was also equilibrated with a large volume of the non-competitive reversible inhibitors, 10^{-5}M mercuric chloride or p-chloromercuribenzoate. However, the granular stroma obtained after centrifugation formed an emulsion with butanol which would not pass through the filter, although the uninhibited stroma gave typical results, (cf. Table 7).

Experiments were also carried out using competitive sugars. The stroma was loaded with either mannose or 3-0 methyl glucose together with the D-glucose and L-glucose. 38.8mg of 3-0 methyl glucose or 36mg of mannose were added to 4ml of the stroma to give a concentration of 50mM in either case. This was in excess of the half saturation concentration for both sugars which has been shown to be 6.0mM for 3-0 methyl glucose and 13mM for D-mannose at 27°C. Although some binding of D-glucose was observed in several experiments, after the addition of the competitive sugar, it was generally of a lower order than that observed with the experiments carried out simultaneously with no competitive sugar. The results, Table 8, give an indication of the variability observed between experiments, and for this reason mean values were not calculated.

These experiments showed that after the butanol-red cell stroma emulsion had passed through a Versapore filter, the insoluble material collected on the filter would retain D-glucose in preference to L-glucose. The degree of binding could be reduced by the addition of competitive sugars. The brown discolouration observed in the centre of the filter indicated that the material collected was the lipoprotein material found at the aqueous-butanol interface after centrifugation of the emulsion. In several experiments no lipoprotein

material or retention of D-glucose was observed on the filter. This can be compared with those experiments where the thin insoluble interface was not obtained after the second centrifugation of the butanol emulsion.

One problem arising when using the Versapore filters was that the filter did not release all the sugar into the phosphor. Hence a filter immersed in phosphor for 24 hours still gave a considerable number of counts when transferred to a clean vial containing phosphor. Variations in the release of sugar from the filter was probably the reason for the lack of reproducibility between experiments.

DETERGENT SOLUBLE PROTEIN

The results obtained from the millipore experiments indicated that an association occurred between protein and D-glucose in the presence of lipids. Experiments were therefore carried out using red cell membranes which had been solubilized in detergent, a procedure which solubilizes membrane proteins and associated lipids. To demonstrate that this extracted material retained D-glucose binding properties, the protein was carefully precipitated in the presence of a mixture of D-glucose and L-glucose.

COMPOSITION

10% Triton X-100 was added to a 1:2 dilution of red cell ghosts to give a final detergent concentration of 1% which solubilized approximately 50% of the membrane proteins and lipids, (Table 9). 95% of the protein was precipitated by an equal volume of saturated ammonium sulphate, and the precipitate was subsequently dissolved in 1N NaOH for protein assay. However, as NaOH was immiscible with the Scintillation phosphor, the precipitated material was redissolved in 1% Triton X-100 and then centrifuged. This allowed 85% of the precipitated protein to be recovered, (Table 9).

TABLE 9 The Composition of Ghosts, Detergent Solubilized Extract and Ammonium Sulphate Precipitate.
(Figures are mean with the number of determinations in parenthesis)

Sample	Protein conc. mg/ml	% protein recovered	Phosphorous conc. mg/ml	% phosphorous recovered
Ghosts	3.061 (4)	100	103.0 (2)	100
Detergent extract	0.842 (18)	55.0	22.2 (2)	43.0
Ppt. from 4ml solb. ghosts in 1% Triton	2.859 (5)	46.7	63.3 (2)	30.0

Note: The % recoveries are based on the composition of the intact ghosts.

Table 9 gives the protein and phosphorous composition at each stage of

the extraction. Phospholipids were associated with the membrane proteins at all stages of the procedure although a little phospholipid appeared to be lost at each stage.

D-GLUCOSE BINDING BY PRECIPITATION WITH AMMONIUM SULPHATE

To estimate the degree of retention of D-glucose binding to the detergent solubilized material, 2.5ml of ghosts were loaded with 1mM D-(^{3}H) glucose and L-(^{14}C) glucose in a similar manner to the millipore experiment. Tris-HCl buffer, (pH 7.4), was added to give a total volume of 4.5ml and the tubes were then allowed to stand for 5 minutes in an ice bucket. The suspension of ghosts were solubilized with Triton X-100 as described in the methods section. 1.245g of ammonium sulphate was added to 4ml of the membrane solution to precipitate the proteins. The precipitate was centrifuged and redissolved in 1ml of 1% Triton X-100. Samples were taken from the original protein solution, (the reaction mixture, RM), the supernatant after precipitation and the redissolved precipitate for Liquid Scintillation Counting. Samples were also taken from the reaction mixture for protein assay. Results obtained from four experiments on the same sample of ghosts are tabulated in Table 10.

TABLE 10 The Distribution of Dpm after Precipitation of the Membrane Proteins with Triton X-100.

Sample	^{3}H dpm/ml	^{14}C dpm/ml	Ratio ^{3}H/^{14}C	^{3}H dpm bound
Protein soln. (RM)	2,052,000	398,000	5.16	-
Supernatant	1,765,000	366,000	5.10	-
Ppt. in 1ml 1% Triton X-100	129,000	24,500	5.29	3,200

The ratio of D-glucose / L-glucose always appeared to be low for the supernatant and high for the precipitate. This low ratio for the supernatant

was partially eliminated by using smaller sample volumes for radioactive counting and was probably due to ammonium sulphate precipitating in the scintillation phosphor and adhering to the walls of the glass vial. However, as the precipitate and original reaction mixture samples were of similar composition, the high ratio for the precipitate was assumed to be caused by binding of D-glucose to the precipitated material. Results from a number of experiments indicated that the majority of ghost preparations gave a binding of D-glucose in the range 0.78 - 2.11 x 10^{-7}g per mg of protein, (mean 1.14), whilst other preparations only bound a little in the range 0.25 - 0.37 x 10^{-7}g per mg of protein, (mean 0.31).

Precipitation using Trichloroacetic Acid

Acids rapidly precipitate proteins from solution but tend to denature the proteins at the same time. Trichloroacetic acid was therefore used to precipitate the detergent solubilized membrane which allowed a comparison to be made between the binding activity found when the protein was denatured and when the protein was relatively unchanged. Simultaneous experiments were carried out to precipitate the protein, one using ammonium sulphate as described above, and the other by rapidly adding 1ml of 25% or 50% Trichloroacetic acid to 4ml of solubilized protein, (Table 11).

TABLE 11 The Comparison of Precipitation using $(NH_4)_2SO_4$ or Trichloroacetic Acid, (TCA).
(Figures mean with range in parenthesis, normalised to the given $(NH_4)_2SO_4$ result).

Precipitant	Ratio $^3H/^{14}C$ IM	Ratio $^3H/^{14}C$ Ppt.	Dpm bound/ml	10^{-7}g glucose bound/mg protein	% binding activity
$(NH_4)_2SO_4$	5.00	5.20	1,290	1.14	100
5% TCA	5.08	5.24	904	0.72	63.2
10% TCA	4.92	5.03	356 (302-412)	0.38 (0.15-0.28)	32.8 (22.9-49.2)

An acid precipitant reduced the binding of D-glucose to the membrane

extract. With increasing strength of the Trichloroacetic acid, further reduction in binding was observed. This indicated that the binding of D-glucose was associated with the membrane proteins, and although denaturation of the proteins did not eliminate the binding it was markedly reduced.

Comparison of D-glucose Binding with Other Proteins

To check whether the observed binding activity was specific to the detergent solubilized material, the experiment was repeated on the membrane proteins solubilized by butanol extraction from red blood cells and bovine serum globulins

Water washed stroma was loaded with 1mM D-(^{3}H) glucose and L-(^{14}C) glucose, and n-butanol extraction carried out. The proteins in 4ml of the aqueous phase were then precipitated using 1.245g of ammonium sulphate. For the second experiment a protein solution containing 1.0mg/ml bovine serum globulin in 1% Triton X-100, 5mM Tris-HCl, (pH 7.4) and 10mM NaCl, was loaded with glucose The protein in 4ml of this solution was then precipitated with ammonium sulphate as before. The comparison between the results obtained for these experiments and those obtained for the detergent solubilized material is given in Table 12.

TABLE 12 The Comparison of D-Glucose Binding between Bovine Serum Globulins and Membrane Proteins solubilized in Detergent and extracted by Butanol.
(Figures mean with range in parenthesis normalised to the detergent soluble proteins)

Protein	Ratio ^{3}H/^{14}C PM	Ppt.	Dpm bound/ml	10^{-7}g D-glucose bound/mg protein	% binding activity
Detergent soluble red cell membranes	5.00	5.21	1,290	1.14	100
Butanol extracted red cell membranes	5.21	5.34	253 (0.1-648)	0.17 (0.00-0.44)	15.1 (0.0-38.9)
Bovine serum globulin	5.04	5.08	448 (268-811)	0.46 (0.23-0.72)	40.7 (20.2-62.8)

Although all three proteins showed a preferential binding for D-glucose,

TABLE 13 The Effect of Inhibitors on the Binding of D-Glucose

(Figures mean with range in parenthesis normalised to the control figures)

Conditions	Ratio $^{3}H/^{14}C$ RM	Ppt.	Dpm bound/ml	10^{-7}g D-gluc. per mg protein	% Binding activity
Control	4.00	5.21	1,290	1.14	100
Phloretin (4×10^{-4}M)	4.82	4.95	1,030 (827-1,370)	0.92 (0.77-1.16)	80.2 (67.4-101.6)
$HgCl_2$(10^{-5}M)	4.99	5.17	890 (418-1,150)	0.76 (0.40-0.96)	66.8 (35.4-84.4)
Mannose (50mM)	4.87	5.08	998 (240-1,330)	0.85 (0.23-1.10)	74.6 (20.1-96.8)
Control	5.36	5.42	362	0.31	100
Mannose (50mM)	5.51	5.50	([-]306 to [+]180)	0-0.18	0-56.1
2-Deoxy D-glucose (10mM)	5.17	5.18	([-]67 to [+]217)	0-0.20	0-64.4

the binding affinity of the detergent soluble protein was in excess of the other two. It was interesting to observe that the bovine serum globulins bound over twice the amount of D-glucose bound by proteins from red cells, solubilized by butanol extraction. This was possibly caused by the errors introduced during calculation, as entrapment of sugars by the precipitated globulins was three times that obtained for the other proteins. Hence a small increase in the ratio $^{3}H/^{14}C$ for the precipitate would give a high degree of binding.

Effect of Inhibitors on D-Glucose Binding

Experiments were carried out to demonstrate whether the degree of binding of D-glucose by the detergent solubilized material plays any role in the hexose transport system. The effect of typical inhibitors and competitive sugars on the binding of D-glucose to the Triton X-100 membrane extract was therefore determined, Table 13.

Inhibition of the ghosts was carried out in a large volume of the competitive inhibitor, phloretin (4×10^{-4}M), and the non-competitive reversible inhibitor, mercuric chloride (10^{-5}M), in Tris-HCl buffer, (pH 7.4). Precipitation with ammonium sulphate was carried out after recovery of the ghosts, loading with glucose and solubilization as before. Control experiments were run simultaneously using uninhibited ghosts. It was evident from the results, (Table 13), that equilibrating the ghosts with phloretin did not necessarily reduce the binding of D-glucose, and in several experiments an increase in the binding activity was observed. However, mercury inhibition always appeared to reduce the binding of D-glucose, but the extent of the reduction varied considerably from one experiment to another.

Addition of competitive sugars, eg. mannose, (50mM), and 2-deoxyglucose, (10mM), to the ghosts loaded with D-glucose and L-glucose, led to a small reduction in D-glucose binding. Variation in the extent of this reduction was again observed. In those experiments using ghosts with a low binding activity, the introduction of the competitive sugars occasionally eliminated

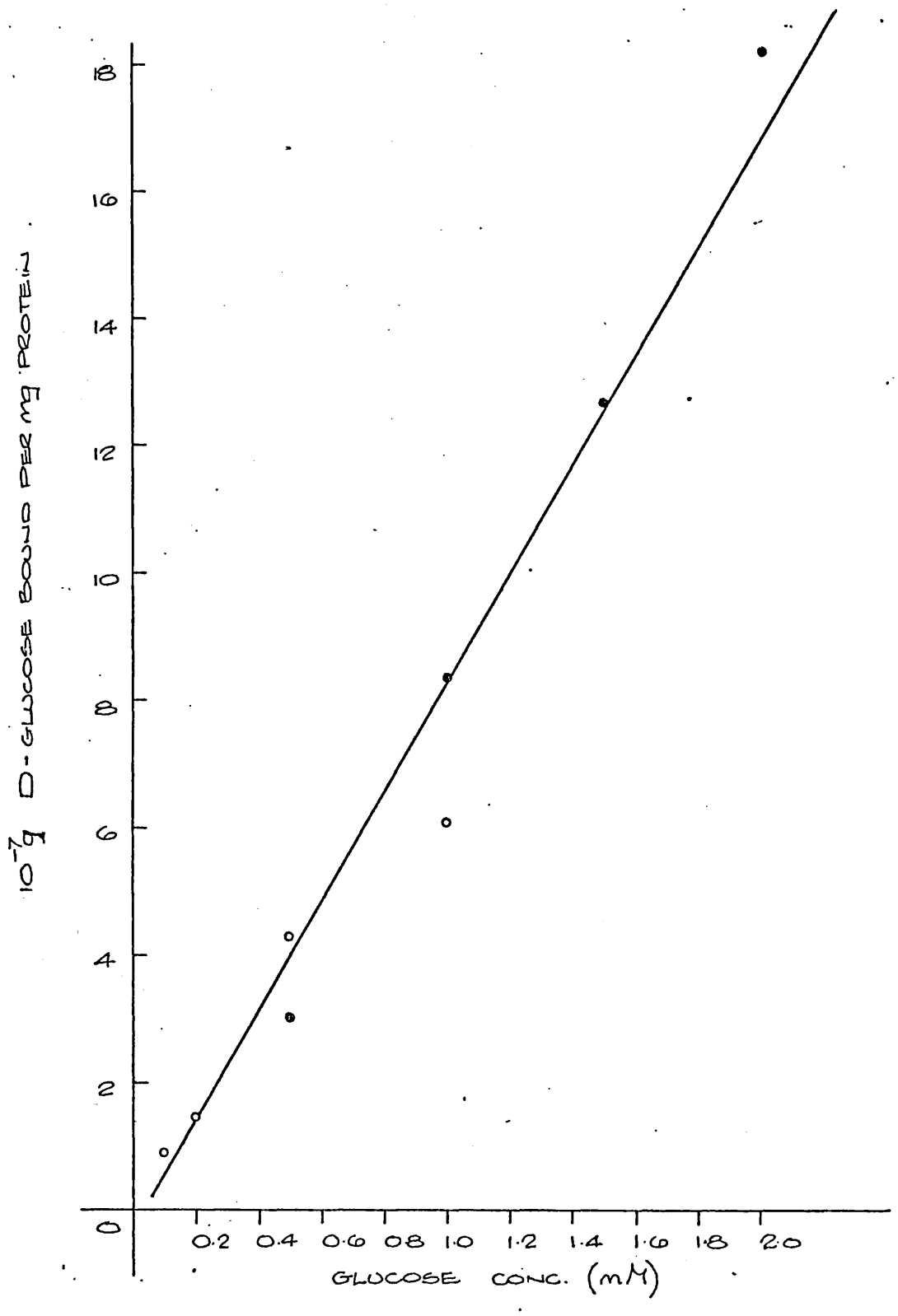


Figure 14 Effect of glucose concentration on the binding of D-glucose to Triton X-100 ghosts.

(The points shown are from two separate sets of experiments).

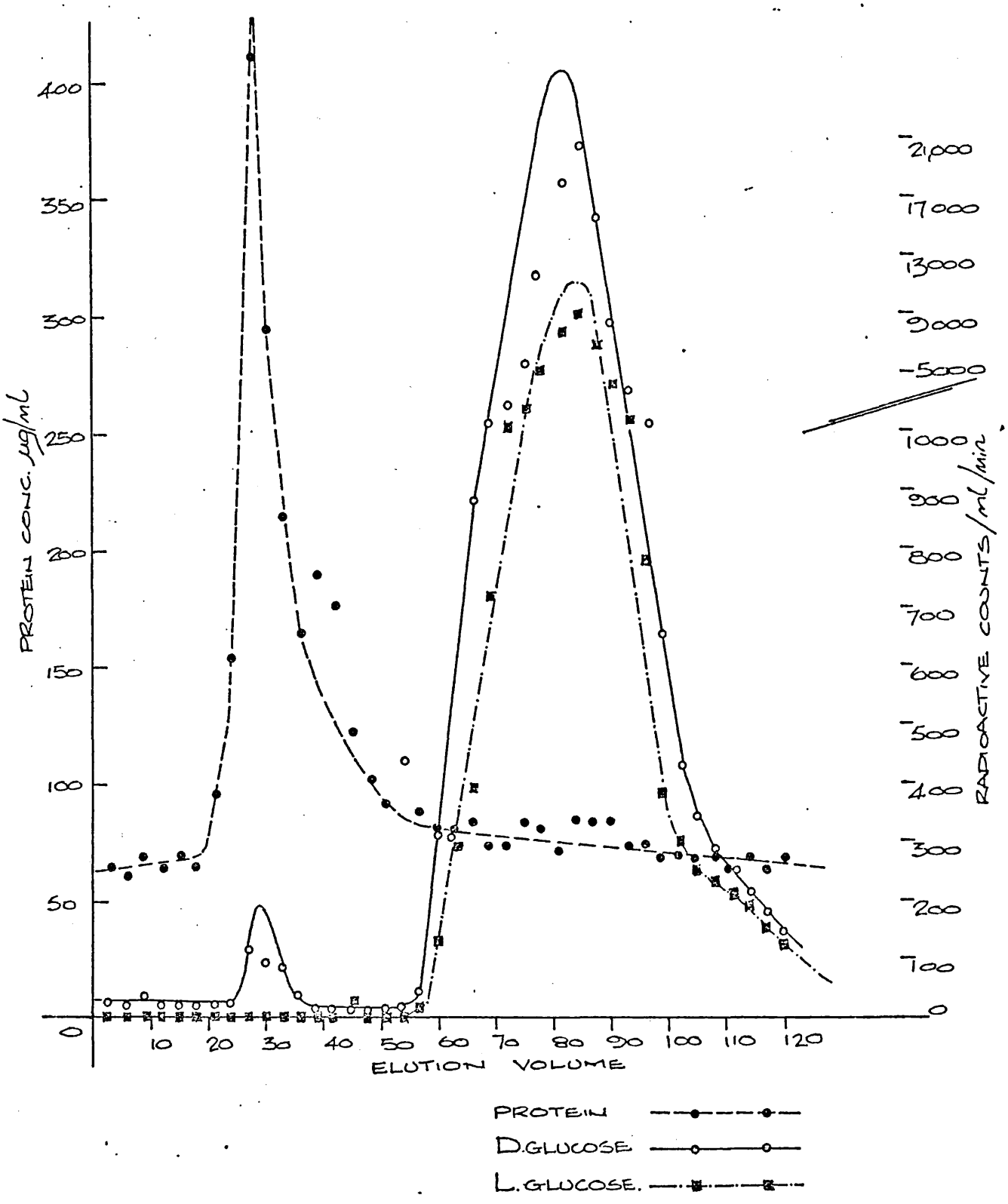


Figure 15 Sephadex G-200 column chromatography (20 x 2cm).
Sample: 10mg of protein incubated with D-glucose and L-glucose
Buffer: 150mM NaCl, 10mM Tris-HCl (pH 7.4).
Flow rate: 7.5ml/hour.

the binding of D-glucose.

Effect of Sugar Concentration on the Binding of D-glucose

Ege, [1919], and LeFevre, [1948], demonstrated that with increasing glucose concentration, the rate of transport of glucose across the red cell membrane was reduced. Hence an experiment was conducted to see if the binding of D-glucose by the detergent solubilized extract showed saturation effects with respect to the glucose concentration.

Sen and Widdas, [1962], demonstrated that the half saturation concentration for glucose transfer was 0.58mM at $7^{o}C$. Thus the binding experiments, carried out in an ice bucket, were performed using a glucose concentration range of 0.1 - 2.0mM. The results of two different sets of these experiments are shown in Figure 14.

It was evident that the binding of D-glucose showed no saturation effects over this concentration range.

GEL CHROMATOGRAPHY

Gel chromatography was used to investigate whether the binding of D-glucose to the detergent solubilized extract was associated with any one particular protein component. Ghosts were loaded with a mixture of D-glucose and L-glucose as in the binding experiments, and then solubilized with Triton X-100. The solution was concentrated by ultrafiltration until it contained approximately 5mg protein/ml. 2ml of the solution was placed on a standardised Sephadex G-200 column and eluted with Tris-HCl buffer, (pH 7.4). 3ml fractions were collected at a flow rate of 7.5ml/hour and 100µl samples taken from each fraction for protein assay by the Lowry method, Figure 15.

The majority of the protein formed a large molecular weight fraction which was eluted in the zone volume of the column. Occasionally a very small fraction immediately followed the elution of the major fraction. The elution patterns of the free D-glucose and L-glucose were identical. However,

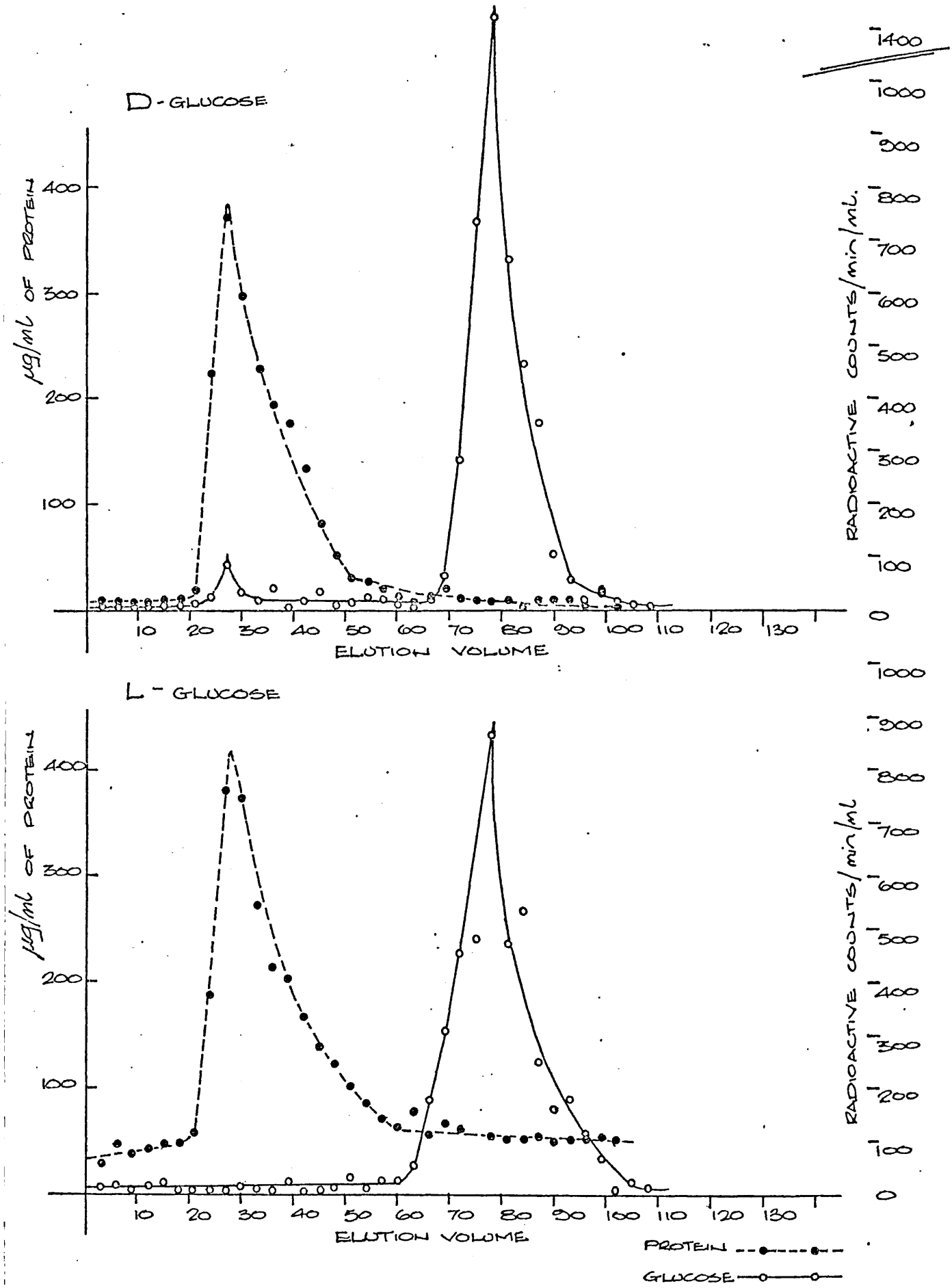


Figure 16 Sephadex G-200 column chromatography (20 x 2cm).
Sample: 10mg of protein incubated with glucose.
Buffer: 150mM NaCl, 10mM Tris-HCl (pH 7.4).
Flow rate: 7.5ml/hour.

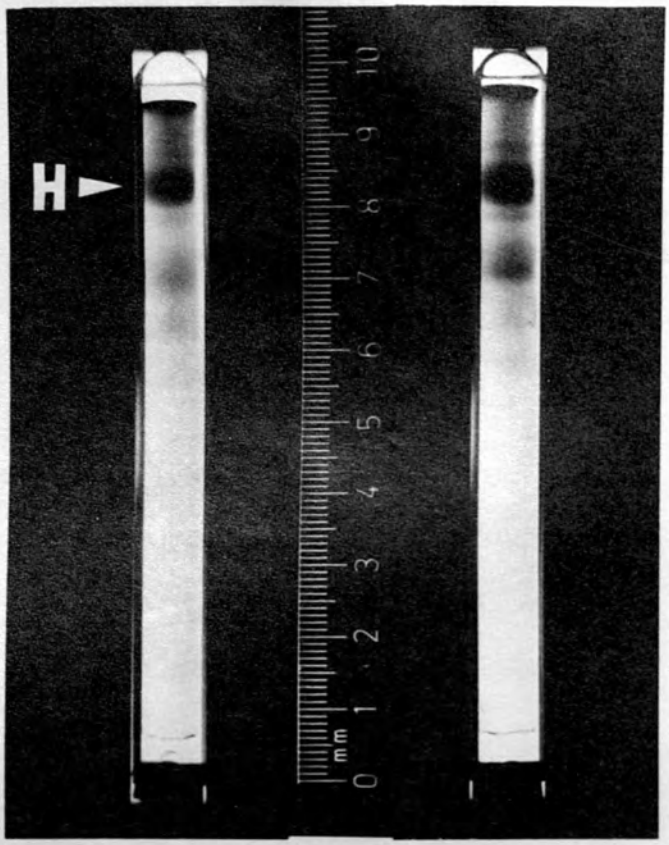


Figure 17 Acrylamide Gel Disc Electrophoresis
Triton X-100 (7%) gels in Tris-HCl buffer, (pH 8.5), 3mA/gel for four hours.

Left: Sample of Triton X-100 membrane extract.

Right: Sample of ammonium sulphate precipitate.

TABLE 14 Radioactive Counts from 5mm Sections of 7% Polyacrylamide Gels in Triton X-100.

Gel (mm)	Triton X-100 Solubilized Ghosts				Ammonium Sulphate Precipitate			
	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$	Incr. ^{3}H dpm	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$	Incr. ^{3}H dpm
RM	139,900	26,800	5.22					
5	21,900	2,600	8.22	7,990	2,350	444	5.30	36
10	1,470	140	10.5	736	123	26	4.74	-
15	253	38	6.63	55	75	19	4.03	-
20	148	25	5.82	17	90	23	3.91	-
25	138	18	7.67	44	51	21	2.43	-
30	128	24	5.36	3	64	17	3.73	-
35	121	8	16.2	79	49	16	2.96	-
40	158	11	13.8	101	47	19	2.53	-
45	52	18	2.99	-	58	19	3.10	-
50	192	41	4.63	-	67	22	3.00	-
55	48	16	3.08	-	50	17	2.89	-
60	36	15	2.38	-	51	23	2.17	-
65	49	14	3.45	-	45	16	2.78	-
70	39	18	2.20	-	40	18	2.30	-
75	77	25	3.10	-	46	18	2.57	-
80	45	15	3.05	-	43	20	2.21	-

D-glucose gave a second small peak, (about 100 counts/ml/minute), compared with the free glucose peak which was eluted in the same sample containing the major protein fraction. This second small peak was not given by L-glucose.

To check that the small D-glucose fraction was not due to overloading the column with radioactive D-(^{3}H) glucose, two separate experiments were conducted on the same Sephadex G-200 column. The sample for the first experiment was detergent soluble protein loaded with D-(^{14}C) glucose and for the second, protein loaded with L-(^{14}C) glucose. Care was taken to ensure that the concentration of labelled sugar was the same in both experiments. No unlabelled sugar was added for these experiments. Glucose and protein assay were carried out on each sample as before, Figure 16. A small amount of D-glucose was again observed to be associated with the protein fraction, but not for L-glucose.

ELECTROPHORESIS

Electrophoresis was also carried out on ghosts solubilized in Triton X-100 and the ammonium sulphate precipitate, to determine any association between specific protein components and D-glucose. Gel disc polyacrylamide electrophoresis was used in preference to the flat gel system because of the association of the proteins in the sample slots. Glucose loaded ghosts, solubilized in Triton X-100, and the subsequent ammonium sulphate precipitate could be characterised on 7% polyacrylamide gels in 0.1% Triton X-100. After electrophoresis, the gels were taken either for staining with Coomassie Blue or 5mm sections were taken for radioactive counting.

A number of bands of different staining intensity were found in a reproducible pattern as shown in Figure 17. The band marked H represents residual traces of haemoglobin in the sample, (as demonstrated by characteristic staining with benzidine peroxide).

Radioactive assay of the gels is shown in Table 14. Glucose entered the first 15mm of the gel which was demonstrated by both D-(^{3}H) glucose and

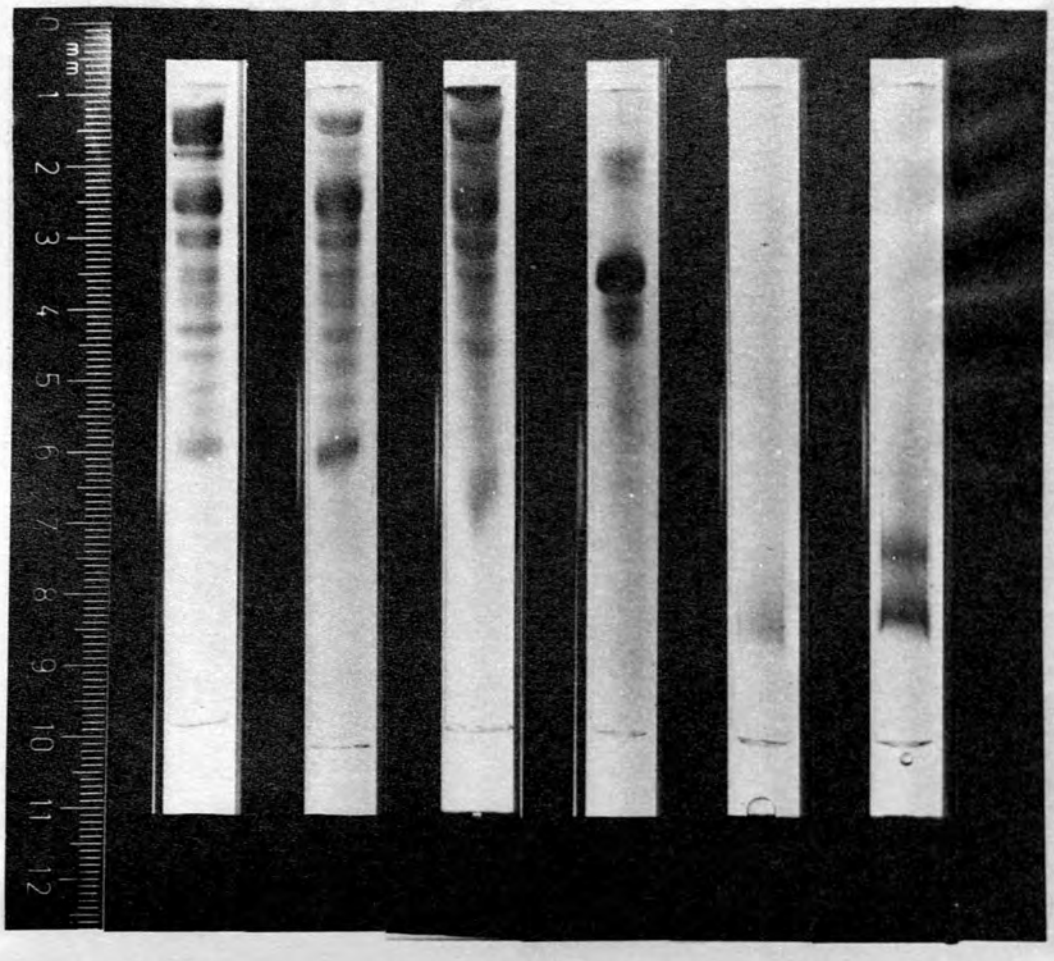


Figure 18 Acrylamide Gel Disc Electrophoresis
SDS (10%) gels in phosphate buffer, (pH 7.0), 8mA/gel for five hours.

Left to Right:

1. Ghosts
2. Triton X-100 extract
3. Ammonium sulphate precipitate
4. Serum albumin
5. Cytochrome C
6. Chymotrypsin

TABLE 15 Comparison of Molecular Weights of Protein Subunits found on SDS Polyacrylamide Gels between the Original Ghosts, Triton X-100 Extract and Ammonium Sulphate Precipitate.

Ghosts M.W. 10^3	Triton X-100 extract M.W. 10^3	Ammonium Sulphate precipitate M.W. 10^3
141.5 - 177	172	195
-	162	164
-	150	155
-	145	146
140	-	-
134.5	-	-
128.5	-	-
95.5 - 117.5	98 - 125	98 - 119
92.5	-	-
85.5	83.5 - 90	81 - 86
80.5	71.5	71
68	67	66
65.5	-	-
57.5	59.5	58
50	49.5	48
43.5	43.3	-
34.8	34.8	36.5
31.8	-	-
26.8	25	22
17.8	-	-

TABLE 16 Radioactive Counts from 5mm Sections of 10% Polyacrylamide Gels in SDS.

	Ghosts in SDS				Triton solb. membranes in SDS			
Gel (mm)	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$	Incr. ^{3}H dpm	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$	Incr. ^{3}H dpm
RM	70,000	14,600	4.80	-				
5	5,970	1,300	4.60	-	5,900	1,060	5.59	834
10	1,060	236	4.47	-	2,680	550	4.87	38
15	105	23	4.56	-	567	116	4.90	10
20	28	9	3.11	-	139	20	6.95	42
25	27	7	3.86	-	118	10	11.8	69
30	16	6	2.67	-	55	6	9.17	26
35	15	5	3.00	-	76	6	12.7	47
40	11	6	1.83	-	30	7	4.57	-
45	11	5	2.20	-	34	8	4.25	-
50	14	4	3.50	-	37	9	4.11	-
55	11	6	1.83	-	26	8	3.25	-
60	12	4	3.00	-	27	9	3.00	-
65	14	5	2.80	-	30	8	3.75	-
70	14	5	2.80	-	63	10	6.30	10

L-(^{14}C) glucose. However, for the sample containing the original solubilized ghosts, a higher percentage of D-(^{3}H) glucose appeared to enter the gel than L-(^{14}C) glucose. No significant increase of D-(^{3}H) glucose was observed from the sample containing the precipitate because of the low initial radioactive glucose present.

10% gels in 0.1% SDS were used to compare the protein subunits in the original ghosts, ghosts solubilized in Triton X-100 and the ammonium sulphate precipitate, which were all incubated in 1% SDS. Figure 18 indicates the similar reproducible pattern of the three samples, and the molecular weights of each fraction, roughly calculated using standard proteins, (Table 15), indicated that the Triton X-100 solubilized ghosts and ammonium sulphate precipitate contained all those proteins found in the red cell membrane. When compared with the Triton X-100 gels, SDS gels increased the fractionation of the protein samples.

SDS gels were also used for ghosts and Triton X-100 extracts previously loaded with D-(^{3}H) glucose and L-(^{14}C) glucose. Table 16 gives the results of the radioactive assay of these gels.

10% SDS gels appeared to allow the entry of L-(^{14}C) glucose in a similar manner to that of the 7% Triton X-100 gels. Using samples of ghosts loaded with glucose and then incubated with SDS, D-(^{3}H) glucose entered the gel in the same proportion as L-(^{14}C) glucose. If, however, the ghosts loaded with glucose were solubilized in Triton X-100 prior to incubation in SDS, additional D-(^{3}H) glucose was found in the first 5mm of the gel.

POLYMER STUDIES

The effects of slowly stirring a dilute protein solution with methyl methacrylate polymers containing carbohydrate side groups were investigated. Two polymers were used, one containing galactose as a side group, and the other containing glycerol. A comparison of the two polymers was made in order to estimate whether any binding of protein to the polymer was due to the side group. The results of slowly stirring 25ml of a dilute protein solution from detergent solubilized ghosts or butanol extracted ghosts is shown in Table 17.

TABLE 17 Comparison of the Binding of Proteins to Galactose Methyl Methacrylate and Glyceryl Methyl Methacrylate.

	Protein solb. in Triton X-100		Protein solb. by Butanol extraction	
	Galactose methacrylate	Glyceryl methacrylate	Galactose methacrylate	Glyceryl methacrylate
Original protein conc.	159µg/ml	159µg/ml	187µg/ml	187µg/ml
Protein conc. after stirring with polymer	131µg/ml	128µg/ml	155µg/ml	151µg/ml
Protein retained by polymer	700µg	775µg	800µg	900µg

The polymers appeared to retain approximately 18% of the total protein and although it was assumed that the major part of this retention did not involve the side groups, the glyceryl polymer showed a small increase in the binding properties. The same degree of protein binding was observed for both protein extracts. It was thought that the galactose of the polymer might be involved in the binding of proteins, so a further experiment was carried out.

The galactose polymer, after equilibrating with protein, was quickly washed with water and then stirred with 10ml of 10mM D-glucose or L-glucose.

TABLE 18 Effect of D-Glucose and L-Glucose on protein bound to Galactose Methyl Methacrylate Polymer.

	Detergent Soluble Protein	Protein Solubilized by Butanol Extraction
Protein retained on polymer	675µg	800µg
Protein removed by L-glucose	570µg	663µg
Protein removed by D-glucose	585µg	650µg

If the galactose side groups did bind any protein specifically, D-glucose would have been expected to release the protein more readily than L-glucose. However, the results obtained showed that there was no evidence of either sugar possessing a greater ability to remove any proteins bound to the polymer.

SECTION 4 DISCUSSION

To isolate and identify those membrane components responsible for the carrier mediated transfer of substrates across various cell membranes has been the aim of a number of research workers during the last decade. Their investigations have involved two major experimental steps. Firstly to extract the membrane constituents in their intact form, and secondly to identify the components of the transport carrier. Others have endeavoured to set up model systems to simulate the *in vivo* situation. Possibly the most satisfactory such approach recently carried out has been to use bimolecular lipid membranes with attempts to reconstitute the carrier function in an *in vitro* membrane structure. Such a method has been used to study the sugar carrier system in red blood cells, [Wood et al, 1968, and Jung, 1971]. However, this method failed to mimic a number of features of the sugar transport carrier function, eg. rapidity, saturation and stereospecificity. This failure led to the idea that the carrier function required membrane proteins in addition to the membrane lipids. This conclusion had previously been drawn from studies using lipids in water-chloroform systems, [Jung et al, 1968, and LeFevre et al, 1968]. To support this idea a number of membrane proteins have been isolated which are reported to be responsible for bacterial carrier transport systems, [Fox and Kennedy, 1965, Pardee, 1966, and Kaback, 1968]. In the work reported in this thesis, experiments were conducted to investigate possible 'protein' reacting site in red blood cells using protein extracts prepared by two different methods. One consisted of proteins solubilized by extraction with n-butanol, and the other contained proteins solubilized by Triton X-100.

The aqueous phase obtained after n-butanol extraction of red cell stroma contained over 80% of the membrane proteins and the remainder was assumed to be distributed between the insoluble interface and the butanol phase. The composition of the aqueous phase which contained the protein and practically

no lipid was in agreement with the results reported by Maddy, [1966], for ox erythrocytes and Rega et al, [1967], for human erythrocytes. However, unlike Rega et al, [1967], the insoluble interfacial material was not observed for every extraction. The membrane proteins in the aqueous phase gave a single component of very high molecular weight on Sephadex G-200 chromatography. However, although acrylamide flat gel electrophoresis was unsuccessful for a complete classification of the membrane proteins due to poor separation, it did indicate that the protein mixture was heterogeneous, consisting of at least three components.

It was concluded during the butanol extraction experiments carried out in the presence of radioactive glucose, that some association of protein and glucose might be occurring in the aqueous phase or the interfacial material. A temporary association of protein and glucose was indicated by using electrophoretic techniques. Protein migration on a cellulose acetate membrane was retarded by glucose, although the simultaneous electro-osmotic movement of glucose appeared to be accelerated. In contrast, protein migration was unaffected by glucose on acrylamide flat gel, but glucose movement, in the same direction as the protein migration, was enhanced. However, as these movements of glucose could be demonstrated with either D-glucose or L-glucose and in the presence of 6M Urea, the protein-glucose associations must have been physical or if chemical they were probably not involved in the sugar carrier function. Although binding of glucose to an 'oxidised lipid' fraction was demonstrated on Sephadex G-75 chromatography, neither gel chromatography nor electrophoresis of the aqueous phase demonstrated any specific binding of D-glucose to a membrane component.

Specific binding of D-glucose, (but not L-glucose), by membrane proteins extracted by butanol was suggested in experiments using a dialysis technique. This binding, however, could be eliminated by carrying out careful sterilisation precautions and was therefore probably due to bacteria introduced during the preparation of the ghosts. These results illustrated a serious problem arising during the study of carrier transport systems in which D-glucose

is the substrate involved. Since D-glucose is an important substrate to living organisms the D-glucose label could be 'fixed' in a large variety of ways if a small amount of metabolism occurred and this label would appear to be due to preferential binding. This source of difficulty is indicated in the results from other authors. Levine and Stein, [1967], found that the retardation of D-glucose relative to L-glucose on celite columns containing red cell membrane extracts, and the binding of D-glucose using an ultrafiltration technique, could both be eliminated by the addition of thymol, (although chloramphenicol only resulted in a slight reduction in binding). Also LeFevre and Masiak, [1971], on repeating the experiments of Levine and Stein, [1967], with celite columns and membrane extracts, could find no retardation of D-glucose except in the presence of a radioactive contaminant.

When comparing the D-glucose binding by different proteins on precipitation by ammonium sulphate, it was observed that those membrane proteins solubilized by butanol extraction, gave extremely low results which were about 40% lower than the binding observed by bovine serum globulins.

The proteins solubilized by butanol extraction, which were in aqueous solution and lipid-free, have become partially denatured with a resulting loss of the specific binding of D-glucose. Considering the evidence of Jung et al, [1968], LeFevre et al, [1968], and Jung, [1971], that lipids could be involved in the carrier mechanism, it was interesting that the insoluble material observed at the oil-water interface during butanol extraction was found to bind glucose. The binding of glucose to this material was specific for D- as against L-glucose, as shown by the retention of radioactivity on a millipore filter. A similar method was used by Eichholz et al, [1969], to study the absorption of glucose by hamster intestinal brush borders. Here too binding of D-glucose, but not L-glucose, was shown by the retention of radioactivity on a millipore filter.

This interfacial material was reported by Zwaal and van Deenen, [1968a], to be a lipoprotein consisting of 15% of the membrane proteins and 5% of the

membrane lipids,

As the results varied considerably between different experiments it was difficult to show whether the binding of D-glucose played any role in the carrier mechanism. No conclusive results could be obtained using competitive and non-competitive inhibitors, but very high concentrations of competitive sugars, (50mM mannose and 3-0 methyl glucose), reduced the binding of D-glucose by approximately 50%. If all the binding was involved in the carrier function it would have been expected that these concentrations of sugars would have largely eliminated binding, but this was only observed in about half the experiments.

The second protein extract used in the experiments were proteins solubilized by the non-ionic detergent, Triton X-100. Triton X-100 is thought to disperse the lipids from the immediate environment of the membrane proteins. Complete dissociation of lipids from the membrane proteins occurs during incubation of red cell ghosts in the ionic detergent, SDS, but it is not known whether the same occurs with Triton X-100, [Dulaney and Touster, 1970]. Although a residue remained after Triton X-100 solubilization, the composition of protein and lipid in the Triton X-100 solution was found to be similar to the composition of the intact membrane. Addition of ammonium sulphate to the membrane solubilized in Triton X-100, precipitated practically all the solubilized lipids together with the membrane proteins. As SDS completely solubilizes red cell membranes, a comparison could be made between the protein subunits solubilized by SDS and by Triton X-100 on SDS-polyacrylamide gels. It was found that Triton X-100 solubilized a full spectrum of membrane protein which could then be precipitated by 50% saturated ammonium sulphate. SDS-polyacrylamide electrophoresis resolved the proteins solubilized by Triton X-100 into 15 - 17 zones ranging in molecular weight from 25,000 - 170,000, which were comparable with the red cell membrane proteins extracted by other techniques, [Rosenberg and Guidotti, 1969, Dennis and Mehl, 1970, and Blumenfeld et al, 1970].

It was found that if the Triton X-100 solubilized material was precipitated with ammonium sulphate in the presence of radioactively labelled D- and L-glucose, the precipitate would retain more D-glucose than L-glucose. This retention of D-glucose was considered to be a specific binding of D-glucose to a membrane component. The degree of binding varied from one experiment to another, so a mean result was calculated of 1.14×10^{-7}g of D-glucose bound per mg of protein. However, with a few ghost preparations, only a low binding activity was observed, ie. 0.31×10^{-7}g of D-glucose bound.

LeFevre, [1961c], estimated that the number of operational sugar carrier sites could not exceed 5×10^{5}, and Stein, [1968], calculated the number of sites per red cell to be 8×10^{5}. If it is assumed that the binding of D-glucose to Triton X-100 solubilized material is all concerned with the sugar carrier function, it can be calculated that the mean result obtained of 1.14×10^{-7}g D-glucose bound per mg of protein represents 95,000 active sites per red blood cell. This number is acceptable as the reported results above probably include some non-specific binding, and a number of active sites were expected to be lost during the preparation of red cell ghosts, [Jung, 1971].

Although the ammonium sulphate precipitate consists of both lipid and protein, the binding component is probably protein, (or lipoprotein). This is indicated when trichloroacetic acid is used instead of ammonium sulphate as the precipitant, and is found to reduce the binding of D-glucose by almost 70%. Binding of D-glucose to other proteins was also observed for membrane proteins solubilized by butanol extraction and bovine serum globulins using ammonium sulphate precipitation. The degree of binding was much lower, 85% and 59% respectively, than the binding of D-glucose to the Triton X-100 solubilized membrane.

Fox and Kennedy, [1965], found that the membrane carrier for the β galactoside transport system in Escherichia Coli was a protein component termed the 'M protein'. They reported that this M protein retained its ability to bind N-ethylmaleimide after the protein had been extracted with

Triton X-100. This membrane protein, (or lipoprotein), solubilized by Triton X-100 could possibly be the surface reacting site postulated by LeFevre et al, [1968].

eg. sugar + surface reacting site (protein?) $\longleftrightarrow$ surface complex (sugar + protein?)

+

phospholipid

$\updownarrow$

mobile complex $\longrightarrow$ (phospholipid + sugar + protein?)

However, although the binding of glucose to the protein solubilized by Triton X-100 was specific to D-glucose, it was difficult to explain certain other results in relation to the properties of the hexose carrier system in red blood cells.

Effect of Inhibitors

LeFevre, [1954], calculated from kinetic experiments that the inhibitor constant for the competitive inhibitor, phloretin, was 4×10^{-6}M at 38°C. However, no conclusive results were obtained which demonstrate a reduction of D-glucose binding to Triton X-100 material at 0-4°C using a phloretin concentration of 4×10^{-4}M.

A reduction in the binding of D-glucose was observed using 10^{-5}M concentration of the non-competitive inhibitor mercuric chloride. However, this reduction was only approximately 35%, although LeFevre, [1948], reported that a 10^{-6}M concentration of mercuric chloride effectively inhibited glucose transport across red cell membranes.

Effect of Competitive Sugars

LeFevre and Davies, [1951], showed that sugars would compete with each other for the carrier according to the affinity of each sugar for the carrier. The affinity constant at 37°C is 7mM for glucose and 20mM for mannose, [LeFevre and Marshall, 1958]. Hence a 50mM concentration for mannose would be expected

to inhibit very substantially a 1mM concentration of D-glucose at 0-4°C. However, mannose only reduced the binding of D-glucose by 25%. Experiments carried out on ghosts with a low binding activity showed that binding could be completely eliminated in a few experiments using 50mM mannose or 10mM 2-deoxy-glucose, (the latter having an affinity constant of 5mM, [LeFevre and Marshall, 1958]).

Saturation of the Carrier System

Ege, [1919], demonstrated that as the glucose concentration increased, the rate of entry of the sugar into the red cell was reduced, and LeFevre, [1948], from similar results, interpreted the results in terms of the carrier becoming saturated. However, over a glucose concentration range of 0.1 - 2.0mM no saturation effects were observed with respect to the binding of D-glucose to protein solubilized by Triton X-100, although the half saturation concentration for glucose at 0-4°C is less than 0.5mM, [Sen and Widdas, 1962].

These results indicate that at least some of the binding of D-glucose is not associated with the sugar transport system, or that certain of its properties are obscurred by the experimental procedures used. One of the major problems to be overcome whilst experimenting with membrane proteins it to obtain a reproducible preparation of the protein extract. Brown and Harris, [1970], demonstrated how small variations in the preparation of red cell ghosts could lead to large differences in the properties of the ghost membrane. Although a standard procedure was always followed in this work, small variations could have occurred possibly resulting in the failure to obtain an interfacial material on every occasion. These small variations occurring during the preparation of the ghost material and the protein extracts could also have been the main cause of the large variations found in the results for the binding activities, both for the interfacial material and the Triton X-100 extracts.

The binding of D-glucose to protein solubilized by Triton X-100 was also demonstrated using Sephadex gel chromatography. The protein subunits tended

to associate on dextran gels to form a single component of large molecular weight. Eluted with this protein fraction was a little D-glucose but no L-glucose. However, attempts to show a specific protein capable of binding D-glucose using acrylamide gel electrophoresis was unsuccessful, although D-glucose appeared to pass into the gel more easily than L-glucose. Generally chromatography and electrophoresis were unsatisfactory as techniques to demonstrate glucose binding because when the sample enters the gel, the concentration of glucose rapidly falls so the 'carrier' molecules would tend to release any bound glucose.

From the results using membrane proteins solubilized by butanol extraction, interfacial material and proteins solubilized by Triton X-100, it appears that the greatest problem is not the isolation of membrane components but the complete identification of the carrier materials. Specific binding of D-glucose to both the interfacial material and membranes solubilized by Triton X-100 has been demonstrated in this work, and future research would necessitate the devising of more sophisticated techniques to ensure that this binding is involved in the sugar-carrier function. These studies indicate that a protein or lipoprotein component binds D-glucose but it would be of great importance to isolate and characterise the specific component involved. This could possibly be carried out by a modification of polyacrylamide gel electrophoresis or by the investigation of specific membrane materials. Two membrane proteins have recently been isolated which may play a functional role within the membrane. Harris, [1969], reported that exhaustive dialysis of ghosts against distilled water produces two proteins; one resembling a hollow cylinder and the other resembling a 'torus' or ring composed of ten protein subunits. If this 'ring' or 'cylinder' had a hydrophilic interior and a hydrophobic exterior it would be possible to envisage a carrier molecule similar to the neutral molecular carrier, monactin, of Eisenman et al, [1968]. Bretscher, [1971], recently reported the isolation of a red cell membrane protein of molecular weight, 105,000, which spans the width of the membrane. A membrane component

traversing the membrane has been postulated by Stein, [1969], and Lieb and Stein, [1971], to account for the carrier properties of the sugar transport system.

The identification of the carrier components would be made easier if these components could be concentrated. The results from polymers containing carbohydrate, although incomplete, did indicate that the polymers could bind protein. The polymers used contained galactose and glycerol as their carbohydrate side groups. Although both these compounds penetrate the red cell by a carrier mechanism, the carrier involved is different in each case, [Bowyer and Widdas, 1956]. The degree of cross linkage of methyl methacrylate to galactose methacrylate was present in the ratio of 9:1, and 4:1 for the glyceryl methacrylate. Hence it would be expected that if the side groups bound specific proteins, more protein would be bound by the glyceryl methacrylate than the galactose methacrylate polymer.

The glyceryl methacrylate polymer bound slightly more protein than the galactose methacrylate polymer, indicating that the carbohydrate side groups are involved in the binding of protein. However, no evidence was obtained that indicated that the binding of the protein by the galactose side group played any part in the carrier function, as the degree of binding was the same for both proteins solubilized by Triton X-100 and butanol extraction, and bound protein could be removed to the same extent with either D- or L-glucose. To continue this work it would be necessary to involve a control experiment using a methyl methacrylate polymer containing a carbohydrate side group which does not penetrate the red cell membrane by a carrier mechanism. Any specific binding of protein due to the side groups could then be identified on polyacrylamide gel electrophoresis. If the polymers could be obtained in a bead form instead of the powder used in these experiments, columns could be constructed, and possibly specific proteins extracted and concentrated by 'column chromatography'.

It now appears that both membrane lipid and protein might be involved in

the carrier mechanism by which substrates are transported across the cell membrane. However, there is still no precise evidence to indicate which membrane proteins, lipids, (or lipoproteins), constitute the 'carrier' and the underlying mechanism by which it operates.

SUMMARY

1. The evidence that sugar transfer across the red cell membrane occurs by carriers is reviewed in the context of recent knowledge of the structure and composition of the plasma membrane, particularly of the red cell membrane.

2. Investigations were carried out on extracts from red cell membranes to examine whether any membrane protein is capable of binding D-glucose, but not L-glucose, and to compare such binding properties with those expected from kinetic studies of the hexose carrier.

3. 80% of the membrane proteins were solubilized by extraction with n-butanol. The aqueous phase containing the proteins was virtually lipid-free. On Sephadex G-200 chromatography only one component of high molecular weight was observed. Horizontal flat gel acrylamide electrophoresis however, indicated a heterogeneous mixture of at least three components.

4. Migration of proteins solubilized by butanol extraction during cellulose acetate membrane electrophoresis was retarded by the opposing electro-osmotic movement of glucose by approximately 10%, although the electro-osmotic movement of glucose itself was accelerated by 3% in the presence of protein.

5. Using flat gel acrylamide electrophoresis the migration of proteins solubilized by butanol extraction was unaffected by a zone of glucose in the path of the protein. Although the glucose moved a short distance of 0.2 - 0.5cm towards the anode under the influence of the protein, these movements were observed using either D-glucose or L-glucose.

6. Apparent D-glucose binding to water washed stroma and proteins solubilized by butanol extraction was demonstrated using a dialysis technique. This 'binding' was found to be due to bacterial contamination and could be eliminated by sterilisation of all solutions and by taking precautions to maintain sterile conditions during dialysis.

7. D-glucose binding to the insoluble material at the oil : water interface during butanol extraction was demonstrated by a technique based on the

retention of radioactivity on millipore filters.

8. The binding of D-glucose to the interfacial material could be reduced to approximately 50% by using high concentrations of the competitive sugars, mannose and 3-0 methyl glucose.

9. Solubilization of red cell membranes with the non-ionic detergent, Triton X-100 yielded a solution of membrane proteins and lipids of a similar composition to the intact membrane. On polyacrylamide gel disc electrophoresis, 15 - 17 zones were found ranging in molecular weight from 25,000 - 170,000.

10. D-glucose binding to Triton X-100 solubilized membranes was demonstrated by precipitation using 50% saturated ammonium sulphate in the presence of D-(^{3}H) glucose and L-(^{14}C) glucose. The approximate figure of 1.14×10^{-7}g of D-glucose bound per mg of protein represents 95,000 active sites per red cell. A few preparations of ghosts gave a lower level of binding of approximately 0.31×10^{-7}g of D-glucose bound per mg of protein.

11. D-glucose binding to Triton X-100 extract was only 30% of the above values if the precipitant used was 10% trichloroacetic acid.

12. On similar ammonium sulphate precipitation of red cell membrane proteins solubilized by butanol extraction and bovine serum globulins dissolved in Triton X-100, the D-glucose binding was respectively 85% and 59% lower than the D-glucose binding to the Triton X-100 red cell membrane extract.

13. Although D-glucose binding to Triton X-100 extract was only reduced in a few experiments using 4×10^{-4}M phloretin, an approximate reduction of 35% was observed using 10^{-5}M mercuric chloride.

14. 50mM mannose and 10mM 2-deoxyglucose reduced binding of D-glucose to the Triton X-100 material by approximately 30%. A more complete elimination of binding using these sugars was only observed in a few experiments.

15. A linear relationship between glucose concentration and D-glucose binding to Triton X-100 extract was observed over the concentration range 0.1 - 2.0mM.

16. D-glucose was also found associated with the protein fraction from Triton X-100 extract during Sephadex G-200 chromatography. No protein subunits were

found associated with glucose on polyacrylamide gel discs, although D-glucose entered the gel more easily than L-glucose.

17. Methyl methacrylate polymers containing glycerol or galactose as side groups were found to associate with membrane proteins solubilized by butanol extraction and Triton X-100. The glyceryl polymer was found to bind approximately 11% more protein than the galactose polymer. But the separation of membrane proteins to concentrate the binding activity was not practicable with the material available.

18. Some of the consequences of these studies to the problem of isolating components responsible for the facilitated transfer of hexoses are discussed and lines of possible further work indicated.

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