STUDIES ON THE GLUCOSE TRANSFER SYSTEM IN ERYTHROCYTES BY INHIBITOR BINDING, AND BY KINETIC AND EXTRACTION TECHNIQUES.

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by

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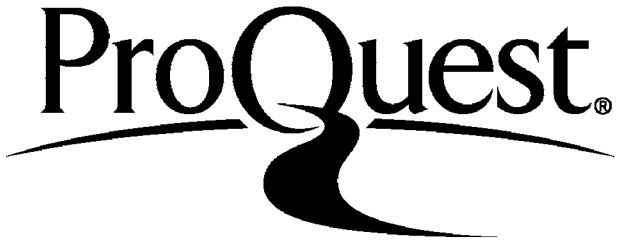

Abstract.

Glucose transport across the erythrocyte membrane is brought about by facilitated diffusion. Extensive kinetic studies have been made of this system, but as yet relatively little is known of the underlying mechanism. Further characterisation of the component(s) involved was attempted.

Various inhibitors and the effects of temperature were studied. Phenolphthalein was shown to be acompetitive inhibitor of glucose transfer. The Arrhenius plot for this substance gave a slope of 18,000 cal/mole while those for phloretin and stilboestrol were 20,000 cal/mole and 4,600 cal/mole. N-phenyl malemide and 1, 5-difluoro-2, 4-dintrobenzene were not found to be more specific inhibitors than their homologues, but the latter compound was used to follow exchange diffusion of sugars in the erythrocyte. Exchange of glucose appeared to occur more rapidly than net flux. Glucose transfer was also shown to be unaffected by sodium concentration in the medium.

Uptake of ^{14}C-labelled n-ethyl malemide and 1-fluoro-2, 4-dinitrobenzene (DNFB) was followed concurrently with the development of inhibition. The two were found to be related, the relationship for DNFB being independent of temperature. A maximum uptake of 400 million molecules per red cell was calculated to give full inhibition. Uptake of ^{14}C-stilboestrol could not be used to give information on the number of sites available for glucose transfer since cell uptake

depended solely on the concentration of the inhibitor in the medium.

^{14}C-labelling from these inhibitors was obtained in butanol extracts, associated with certain lipid fractions, the DNFB being present in a quantity representing 10-20 million molecules per red cell. Several DNP-derivatives were isolated from these fractions.

Erythrocytes and erythrocyte ghosts were extracted, after incubation with ^{14}C-glucose, by various solvents including butanol and isopropanol:chloroform 11:7 to see if evidence of a glucose lipid complex could be demonstrated. Labelling associated with lipid was obtained with several of the solvents in the fractions eluted from silicic acid columns with chloroform:methanol 4:1 and 1:4, free glucose being eluted by chloroform:methanol 3:2.

CONTENTS.

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Abbreviations.

In general the abbreviations used are those recommended by the Physiological Society in "Suggestions to Authors" in The Journal of Physiology (1966) vol. 182 pp 1-33.

Other abbreviations used:-

NEM - N-ethyl maleimide.

NPM - N-phenyl maleimide.

DNFB - 1-fluoro-2, 4-dinitrobenzene.

FDNFB - 1, 5-difluoro-2, 4-dinitrobenzene.

DMF - dimethyl formamide.

TLC - thin layer chromatography.

CHAPTER 1.

Introduction.

General

Παντα ρει - all is flux - nothing remains still and so it is with life, ever changing, yet ever constant. It is part of the uniqueness of life that in an ever changing environment a more or less invariant composition is maintained quite distinct from the surroundings. On this fact Bernard based his famous dictum 'la fixite du milieu interieur c'est la condition de la vie libre', a **dictum** which to a large extent meets the need for a general principle in physiology. A number of elaborate processes exist for the regulation in a species of its internal environment, the overall mechanism was called homeostasis by Canon in 1932. The regulation extends to the smallest biological unit the cell, unless one entertains the idea of an operon and at this level the cell membrane which is rarely indifferent to the bathing medium plays an important controlling role. The membrane may be thought of as a 'black box' regulating flow into and out of the cell.

By 1835 the cell was beginning to emerge as an entity possessing a life of its own and a complex structure and in 1838 Schleiden and Schwann formulated the cell theory. About 15 years later Nägeli as a result of his experience with cryptogams recognized that the protoplasm at the surface was more dense and more viscous and he called it the 'plasma membrane'.

As a result of Nägeli's discovery of plasmolysis and deplasmolysis in 1855 many botanists became interested in the

permeability problem - especially Pfeffer. Pfeffer studied the entrance of water into plant cells at virtually constant volume and finding that some cells behaved in a manner akin to that of an artificial osmometer was also led to postulate an invisible plasma membrane. Thus the existence of a plasma membrane was inferred from an indirect observation as Harvey inferred the capillary bed.

Other workers on permeability at the time did not feel the necessity to invoke a plasma membrane. De Vries studying the escape of water from plant cells attributed the osmotic results to the properties of protoplasm as a whole. Overton after 10,000 experiments in 9 years on the rate of entry of solutes into living cells merely wrote of an "impregnation" of the cell surfaces by substances such as lecithin and cholesterol which he believed to be also present in the cell interior.

The concept of a plasma membrane met great resistance from the colloid chemists. Fischer and Moore for example held that imbibtion by colloid is capable of explaining the phenonomena for which a semi-permeable membrane was postulated. It is only fair to point out, however, that at this time there was no real evidence that there was a fundamental difference between the surface layer and the interior of the cell.

Evidence in favour of a plasma membrane was building up and by 1914 the idea was generally accepted. Apart from the osmotic evidence there were two main lines of evidence at this time. The first was the difference between the cell and the surrounding medium

as regards the presence and concentration of electrolytes. The erythrocytes of rabbit were found by Abderhaldan to contain more potassium than the plasma and no sodium. Höber maintained it was impossible to account for this constant difference in the ratio of potassium to sodium except on the hypothesis of complete semi-permeability. The second was the resistance of living cells to the passage of electric currents through them. This does not necessarily indicate that the membrane is impermeable to salts. If the electrolytes were bound no current could pass. Höber, however, in 1910 and 1912 successfully demonstrated the presence of free electrolytes in the interior of living cells.

The plasma membrane was first given form by the micromanipulative studies of Chambers. By 1922 he had developed a technique by which a drop of aqueous solution could be introduced into the interior of the cell. By use of coloured solutes it could be demonstrated that while there was free diffusion within the cell the substances failed to cross the surface of the cell. Further evidence came from the work done in the early 1940's on membrane ghosts obtained from erythrocytes by lysing them and washing extensively with water. Finally the actual structure may be seen under the electron microscope in the section of a cell stained with a reagent such as osmium tetroxide when the plasma membrane is visible as a dark line at the periphery of the cells.

The Nature of the Plasma Membrane - Development of a Model.

Once the existence of the plasma membrane had been established the next step was to determine the manner in which it exerts its regulatory influence. Studies on artificial membranes up to and at the turn of the century had led to certain mechanisms being proposed to explain the permeability of membranes. Studies of Traub on copper ferricyanide membranes and the work of Walden (1892) on a large number of precipitation membranes gave rise to the idea of membranes acting as molecular sieves. Another suggestion was that certain substances may cross the membrane because of solubility in the membrane or because of the formation of reversible chemical compounds with some membrane constituent. A membrane of palladium, as investigated by Ramsay (1894) was considered to be permeable to hydrogen but not to oxygen, either because the hydrogen dissolved in the palladium or because a reversible compound of some kind formed which dissociated under low tension of hydrogen. These type of studies though laying foundations for further work were inadequate to explain complex biological mechanisms. Not until the chemical groupings are known may a mechanism for transport be defined. It is, therefore, essential to know the composition, dimensions and configuration of the membrane involved.

The chemical composition of cell membranes has been the subject of much discussion. It was known for a long time that

lipoid substances and cholesterol are found in protoplasm and, as they have the power of lowering surface tension, must form an important part of the membrane. In 1889 Overton advocated the idea that access to the cell was limited by a layer of a lipoid nature. The lipoid nature of the membrane was supported by the fact that in starvation a certain proportion of the lipoid content of the body remains relatively constant (the 'element constant' of Terroine) suggesting that some lipids perform a structural rather than a metabolic function and later chemical analysis supported this hypothesis.

While it was evident that lipids formed an important part of the constituents of the cell membrane it was becoming obvious that they were not the sole substances involved if only for the reason that it was difficult to understand how the permeability could be regulated unless complex processes were involved. The presence of more than one constituent in the erythrocyte membrane was early shown by the experiments of Ryvosh (1907) on haemolysis by saponin and according to Pasculli (1905) the stroma of the erythrocyte consists of protein, lecithin and a cerebroside. Mills (1912) showed that red blood corpuscles behaved to the agglutinating action of trivalent cations as if treated with an emulsoid colloid suggesting a protein constituent. Further evidence that proteins are involved in the structure is the fact that proteins are known to be readily adsorbed onto a surface. The very low surface tension of the cell

surface, of the order of Ø,Oidynes/cm^2 (Harvey 1931, Cole 1932) is indicative of a complex structure as no free lipid surface could have such a low value. Another attraction of the protein theory was the sieve-like character of the membrane which was thought to be best provided by polypeptide chains of proteins, but workers were unable to obtain protein membranes which sufficiently resembled the cell structure. Harvey (1912) experimented with protein membranes formed by an adsorption process and Collander in 1927 prepared a membrane fixing gelatine with formalin. These membranes failed to show preferential lipid solubility. Interest, therefore, seems to have centred on lipids.

The developing understanding of cell membranes was to a large extent conditional upon the work of Langmuir on the arrangement of molecules at surfaces. He used many widely different methods, - all with a common inspiration that molecules have a shape, but his chief work was with liquids in the trough. He made many studies covering water with a monolayer of lipid. The typical lipid molecule has one or more paraffin chains and a "head group" - usually polar in nature. An essential feature of his approach was that the molecule should be orientated so that the polar group was located in the water surface and the hydrocarbon tail should project from the surface. From the area of closest packing and the weight of the molecule, the area per molecule could be determined. This work was developed and extended in the decade 1925-35 by N.K. Adams who conducted systematic studies of monolayers, with not only the

idea of molecular shape but also the conception of the orientation of a heteropolar molecule at the surface interface. Studies of monolayers were insufficient to find the actual shape of molecules. It was the X-ray crystallography of Sir William Bragg that showed that the form of the tail was a zig zag. Studies of monolayers also allowed molecular associations to be studied. Important work on these lines was done by Paule Collet (1922), Leathes (1925), Adam and Jessop, and Schulman and Hughes (1933). One important observation arising from studies was the condensing effect of cholesterol. In 1925 Gorter and Grendel extracted lipids from human erythrocytes by means of acetone which was evaporated off later; the residue was dissolved in a little benzene and spread on a Langmuir Trough. They found that the cell membranes contained sufficient lipid to form a membrane two molecules thick. They also enquired into the nature of the lipid molecule. Earlier analysis had shown that from 1 l. blood, 0.5g sphingomyelin, of 0.5g cephalin, lecithin, and cholesterol could be obtained. The relative contributions in terms of spread area were cholesterol 30%, cephalin and lecithin 50% and sphingomyelin 13%. From this they estimated a double layer with average thickness 31A°. The lipid molecules according to this theory are arranged radially. Schmidt et al (1936, 1938) examined the birefringence of the red cell ghost with polarised light and also came to the conclusion that it contained radially arranged lipid molecules and was no more than a few molecules thick. Similar views were expressed by Dzieman (1939) except that he considered the lipids to be in the form of

lipoprotein.

Thus by the late 1930's sufficient evidence on the orientation of molecules had been accumulated for several important theories of membrane structure to be advanced. They had to take into account many conflicting theories of permeability but the three main characteristics of protoplasmic membrane to be accounted for were that it is a statistical sieve, a solvent for substances poorly soluble in water and an ion exchanger. Wolpers (1941) suggested that the lipid was present in patches and Winkler (1938) proposed a unimolecular membrane. The two models which have laid the basis for current concepts are the bimolecular layer of Bungenburg de Jong and the paucimolecular layer of Davson and Danielli. Bugenberg de Jong et al (1935, 1937), as a result of work on simple systems such as gum arabic gelatin coascervates, suggested a double film model consisting of phosphatides in contact at their head groups, together with a thickening substance such as cholesterol. They modified this concept so that the membrane was seen as being a tricomplex between protein, phosphatide and a cation such as calcium. The Davson-Danielli model was first proposed in 1935. The membrane is seen to consist of one or more double layers of lipid molecules which are orientated parallel to one another and at right angles to the plane of membrane with the tails of the lipid molecules in contact with one another. Bound to the polar groups at the surface of the membrane by electrostatic forces are layers of protein which are considered

to be globular. In the early versions of this model the two layers of phospholipid were assumed to be separated by a layer of neutral lipid. This space was eliminated in a later version of the model (1952). This model gives a membrane 80Å in diameter, the two protein layers being 10Å thick and the double lipid layer 60Å thick. They showed that the rate of penetration by organic molecules is what would be expected for penetration of an approximate bimolecular leaflet of lipid molecules by a process of activated diffusion. At the time this molecular arrangement was proposed, information on the thickness of the membrane was produced by Waugh and Schmitt (1940) with the analytical leptoscope. This method was based on a comparison of the intensity of light from dried erythrocyte stroma with that reflected from barium stearate films of known thickness. Their results suggested a thickness of 100Å.

Most of the subsequent work on membranes and membrane models has served to consolidate the picture put forward by Danielli and Davson. Up to the early 1950's information about the molecular structure of cell membranes had been obtained by integrating information acquired in 3 main ways:-

1. Analysis of cell fractions consisting mainly of membranes and the physicochemical properties of their chemical constituents;
2. Studies on the permeability properties of living cells;
3. Analysis of cell membranes with the light microscope, not discussed here.

Other useful approaches seem to have been studies using lytic agents and narcotics. As mentioned, studies of the monolayer had given results but its usefulness as a model must be questioned. It is unlikely that similar planar structures exist in cells and penetration at air water interfaces will be different from that through cell membranes. Still it seems quite a useful system especially for studies related to the mechanism of haemolysis.

As is so often the case with physiology, advances were attendant upon developments in other fields - namely those of physics and chemistry. Recent studies have involved X-ray diffraction and polarization studies of the myelin sheath and electron microscopy. Schmitt and Palmer (1940) who pioneered the work on diffraction patterns suggested that the nerve sheath consisted of concentric plasma membrane lipid leaflets with a repeating unit of 63Å. Finean after more exhaustive studies found a large number of atomic groupings repeated at 85Å. After modifying the spacing by drying, freezing and lipid extraction they were able to conclude that the hydrated protein layer was 30Å thick leaving the lipid layer 55Å across. Their results thus confirm the general picture of the cell membrane.

The laminar arrangement of myelin was confirmed by the electron microscope studies of Fernadez-Moran (1952) and Sjöstrand (1953). Much work with the electron microscope has now been performed on sections of cells from many organs. A large number of preparations stained with osmium tetroxide or potassium permanganate show a dark

line at the periphery of the cell. Under higher magnification a triple layer is seen. Early in the history of electron microscopy it was seen that cell organelles like the mitochondria had complex membranous structures and hosts of canals and vesicles were to be found in the cytoplasm between these organelles. Triple layered structures have now been found at the cell surface and in all these membranous organelles. This led Robertson to propose the unit membrane hypothesis which is very similar to the Davson-Danielli model but has the number of bimolecular leaflets restricted to one and unlike the Davson-Danielli model, is considered to be asymmeterical with mucopolysaccharide or mucoprotein on the outside and conjugated protein on the inside. The protein layers are visualised as polypetide chains note more than 10Å thick. Very recently Green and Perdue (1966) have defined membranes as vesicular or tubular systems made up of a single layer of repeating units which are the only structured elements in the membrane. The units are described as consisting of a base piece which is the membrane forming sector and a detachable sector that is not essential to the membrane continuum.

Another approach to the problem of the orientation of phospholipids within the membrane is the study of phosphlipid/water systems. It has been known for some time that suspensions of cholesterol and lecithin in water formed myelin figures. Subsequent studies on lipiprotein extracts and lipids alone using X-ray diffraction and electron microscopy of preparations fixed and stained with

potassium permanganate and phosphotungstate confirmed that the formations consisted of tubes of bimolecular leaflets, suggesting that in water saturated with phospholipids the bimolecular leaflet is the lowest free energy configuration for undissolved lipid. In some cases, however, the picture obtained was not consistent with a lamellar structure although the observations seem to have been interpreted in these terms. Luzzati (1962) using X-ray diffraction studies and Stoeckenuis (1962) using electron microscopy both concluded that in soap/water systems a micellar structure could exist. Bangham and his co-workers confirmed this and indicated that the lamellar structure of lecithin was controlled by the electrostatic environment at the water lipid interface as Haydon and Taylor (1963) had suggested. Bangham et al (1962,1963,1964) used this system to study the effects of ions and various surface active agents. They found that in a three component system of lecithin, cholesterol and saponin, which was negatively stained, a hexagonal structure appeared similar to that described by Lucy and Gluaert (1964). It has been suggested that similar phase changes may occur in the natural membrane which may be of significance.

The search for a suitable membrane model continued but direct experimental studies of the physical properties of a symmetrical bimolecular membrane in an aqueous phase were not possible till very recently since, apart from their postulated existence in natural membranes, films of this type were unknown. Mueller et al (1965) described the reconstitution "in vitro" of membranes

60Å-90Å in thickness derived from a complex mixture of lipids. The membranes, derived by a process analogous to the formation in air of black soap films, were essentially bilayer structures but were of a complex nature so that the essentials of molecular structure were obscure. They exhibited electrical properties similar to those of natural membranes. Hanai, Haydon and Taylor (1963) and Huang and Thompson (1964,1965, 1966) have prepared membranes consisting of, for example, lecithin and n-tetradecane separating two aqueous phases and with dimensions of 48Å and 61Å respectively, which is compatible with a bilayer structure. These membranes show certain permeability properties characteristic of natural membranes. Hanai et al (1965) have also studied the effects of adsorbed protein on the membrane.

The Erythrocyte - a suitable system for study.

There are many limitations to the study of the lipid composition of membranes, the chief of which are the difficulties in obtaining non-contaminated fractions in sufficient quantities and difficulties of analysis. The first has been overcome largely by the use of erythrocytes. These cells devoid of many of the living constituents serve as simplified models. They are circular biconcave discs with a diameter of 7-8μ, thickness of 2.2μ, a mean volume of 90cu.μ. and a membrane thickness of 60Å. Extractions have been made of intact erythrocytes or of "ghosts" which are the erythrocyte stromata remaining after the cells have been haemolysed by suspension in distilled water or a relatively dilute salt solution. While no

method of haemolysis can produce a stroma which accurately represents the true cell membrane as it is constituted in the intact cell, the stroma does represent a derivative of the original membrane, a study of which can produce a biochemical understanding of the nature of the membrane. The permeability properties of the ghost are not too altered. Teorell (1952) showed that untreated ghosts behave as almost perfect osmometers being semi-permeable in the sense that K^+ and Na^+ diffuse slowly while water is rapidly transferred across the ghost membrane. Lefevre (1963) demonstrated the persistence of carrier mediated sugar transport in the erythrocyte ghost.

Biochemical Methods of Study.

Only with improved techniques has the widespread analysis of phospholipids been possible. In the last fifteen years or so the traditional techniques of lipid fractionation, such as fractional crystallisation, have been almost completely replaced by chromatographic methods. The term chromatography had been used for some time before Tswett coined it for his special application. (It had been used to define a treatise on colour). Williams (1952) proposed a definition 'by chromatography is meant those processes which allow resolution of mixtures by effecting separation of all their components in concentration zones or in phases different from those in which they are originally present, irrespective of the nature of the forces causing the substances to move from one phase to another'. At present the three chief ways of separating material between a matrix

and a solvent or two solvents are on paper, in columns or on thin layers. The first person to seriously consider the phenonmenon of separation due to capillary action was a dye chemist F.R. Runge in the first half of the nineteenth century who separated dyes and inorganic cations on paper. Runge was followed by Goppelsroeder, who introduced what is now known as the "Rf factor", a measure of the rate at which disolved substances move relative to the solvent when the solution spreads in porous matter.

The next major development came from interest in chromatographic analysis on columns of adsorbents. The origins of column chromatography go back to the middle of the nineteenth century. Early investigators like Malleucci and Goppelsroeder believed that the separation was a result of the same capillary forces as assumed to operate in the lattice of the paper. It is now realised that they are the result of differences in the adsorbtive affinity of the constituents of the mixture for the solid phase of the column. Further advances came from soil chemists and industrial chemists such as D.T. Day (1900), who separated the various constituents of natural petroleums on columns of Fullers' Earth. Important experiments on adsorbtive chromatography were made by Tswett (1903), He was able to separate extracts of green leaves in petroleum ether into a number of coloured bands by pouring extracts on to a column of powdered calcium carbonate. Despite Tswett's extensive work on the subject, the process was virtually ignored for thirty years until Kuhn and his co-workers in 1931 developed the technique for the separation of polyene pigments.

The next step forward came when Martin and Synge (1941), introduced partition chromatography. It had been known for some time that a many stage fractional distributioncould be used to separate a mixture whose components differed but slightly. Martin and Synge realised that a multi-stage process could be achieved in another way, distribution occurring between two liquids in a silica gel column. One liquid represented the mobile phase and the other the stationary phase, which was held in the pores of the silica gel. This method appears to be the same as that of Tswett, but uses different principles.

Since then has followed a period of rapid development in column chromatography incorporating techniques of frontal displacement analysis, carrier displacement and gradient elution analysis. The use of silica columns, however, could not meet all their needs and in 1944 Martin and his co-workers introduced two dimensional chromatography on paper. Leisegang had already introduced the idea of using strips in order to gain extra space for manoeuvring the substance to be separated, thereby preparing the way for two dimensional chromatography.

Another important branch of chromatography is thin layer chromatography. Tswett's column chromatography had been successful but the methods could not be successfully adapted for micro-separations. The problem was solved by using an open column - thin layer separation. Thin layer chromatography is of special use in separating lipophilic

mixtures as it is much more convenient than separation on impregnated papers. As early as 1935 the basic principle underlying the process had been observed by Izmailov and Shaiber who observed the division of one drop of substance into zones on thin layer of adsorbent. Twelve years ago Stahl gave a detailed description of the separation of substances on thin layers of very fine grained silica gel, but it is only in the past five years that thin layer chromatography has been widely adopted.

Lipids of the Erythrocyte Membrane.

With reliable separation methods available, improved extraction procedures have been developed. Dawson (1960) introduced a method for identifying phospholipids which is now standard. The material is hydrolysed with mild alkali, the water soluble products are separated by two dimensional chromatography and estimates made of the phosphorus content of the esters. Extensive and accurate work has been carried out on the analysis of cells and "ghosts" by Dawson, Hemington and Lindsay (1960), Hanahan, Watts and Papjohn (1960), Reed, de Gier, Van Deenen, et al (1961), Matsumoto (1961), Axelrod, Reichenthal and Brodie (1953), Formigne and Poulie (1957).

Of the lipids of the erythrocyte some thirty per cent are neutral lipids, cholestrol with some glycerides and other non-phosphorus containing chloroform-soluble material. The remainder is chiefly made up of phospholipids which can bear charges. Hawthorne has suggested that phospholipids be classed as glycerophos-

phatides and sphingophosphatides. Glycerophosphatides derive essentially from phosphatidic acid which is composed of a glycerol molecule esterified with two fatty acid moities and phosphoric acid at the terminal alpha-hydroxyl group. The phosphoric acid may be bound by an ester linkage to choline, ethanolamine or L-serine to give phosphatidyl choline etc. The lyso form of the compound simply contains one fatty acid residue and in the plasmalogen the fatty acid at the alpha position is replaced by an alph-beta unsaturated ether. The remaining glycerophosphospholipids contain no bases. Sphingolipids as sphingomyelin containing choline and cerebroside containing D-galactose derive from the nitrogenous base sphingosine.

The chief phospholipid components of the human erythrocyte appears to be phosphatidyl choline (lecithin) and its lyso compound, phosphatidyl ethanolamine (cephalin) phosphatidyl serine and their plasmalogen forms also sphingomyelin. Small quantities of phosphatidic acid and phosphatidyl inositol are found and there is some uncertainty as to whether diphosphatidyl glycerol (cardiolipin) is present. The various authors differ somewhat as to the percentages to be found. In the preparation of "ghosts" essentially all the lipid is recovered, but several authors have reported that "ghosts" contain slightly more phosphatidyl serine and slightly less phosphatidyl ethanolamine..

With the advent of gas liquid chromatography it has become possible to perform a rapid analysis of the fatty acids of lipids. The methyl ester of the fatty acid is volatilised and swept through a

column of a finely divided inert solid such as diatomaceous earth by a constantly flowing stream of an inert gas. Each component of the ester mixture moves at a rate determined by its ratio of partition between the gas phase and the non-volatile liquid (stationary) phase. Various chemical and physical means are available to detect the ester. It has been found that while individual phospholipids differ in fatty acid composition the relative proportions of fatty acids tend to be characteristic of a certain phospholipid in individual tissues.

Comparative studies have been made on the phospholipids and fatty acid composition of red cells of various mammalian species by de Gier and Van Deenen. Turner (1957) earlier had been unable to find any lecithin in the red cells of ruminants. De Gier and Van Deenen (1961) found that erythrocytes of the animal series rat, man, rabbit, pig, ox and sheep revealed a significant fall in the proportion of lecithin to sphingomyelin in this order. The percentage of arachacidonic and palmitic acids in the total phospholipids fell whereas the oleic acid content rose in the given sequences. The permeability of the red cell to glycerol also decreases in the same order. The suggestion that the permeability of erythrocytes might depend on the lipid components had been made by Höber (1935) and Dzieman (1939). After experiments involving alterations in dietary fatty acid it was concluded that differences in fatty acid composition could not account exclusively for the distinction in permeability behaviour of red cells from various animal species.

Tunrover of Phospholipids in the Erythrocyte.

These dietary experiments also gave an indication of the factors controlling phospholipid metabolism. While it appears that pertinent variations are to a certain extent brought about by dietary variation, the specific phospholipid composition is probably governed by genetic factors. There is evidence suggesting that the lipid fraction of the membrane exists in a dynamic state, although the protein does not do so. It was, therefore, of interest to see the extent of phospholipid synthesis in the red blood corpuscle. The red cell does not on the whole maintain many metabolic processes, which is one important factor in its use in permeability studies. Altman (1951) and Lovelock and Webb (1957) have shown labelling of lipid occurs if the blood is incubated with ^{14}C-acetate and that the labelled lipids are then released in to the plasma. Buchanan (1960), however, has shown that the red cell contribution must be very small and Van Deenen has suggested that there might be no net synthesis of phospholipid in the mature erythrocyte. Many authors have shown that there is an extensive transfer of fatty acids and sterols between human alpha-lipoprotein and erythrocytes. From the work of Mulder and Van Deenen (1965) it appears that the phospholipid constituents are renewed by complete exchange of entire phospholipid molecules and enzymatic transacylation. This second process is mainly responsible for the uptake of fatty acids into the phospholipids of intact erythrocytes, thus the mono-acyl derivatives as well as

the fatty acids are provided by the serum. Robertson and Lands (1964) have found an active acylating system in the human erythrocyte with a rate of synthesis of phosphatidyl choline several times that of phosphatidyl ethanolamine. The incorporation of phosphate of phospholipid appears to be of a low order, the incorporation of phosphoglycerate into phospholipid falling during maturation of the erythrocyte. Robertson also feels that a significant factor controlling erythrocyte phosphatide composition may be differences in the protein portion of the cellular lipoprotein that leads to a more stable binding of one phosphatide over another.

Protein and Lipoprotein Complexes in the Erythrocyte Membrane.

While the lipids of the erythrocyte stroma have been well characterised, inadequate description has been made of the protein content. Studies of stromal protein have been hindered by difficulties in solubilizing the protein. Maddy (1964) achieved this by extracting a suspension of erythrocyte "ghosts" with butanol. After centrifugation in the cold at 20,000g. for ten minutes the lower aqueous phase containing the protein could be removed. Ninety per cent of the protein could be recovered as a single component with a molecular weight of 200,000. The bulk of the protein is strongly acidic and it is suggested that this is due to the sialic acid residues. By using a fluorescent label which reacts only with the outside of the cell, it has been possible to demonstrate that much of the protein resides on the outside of the permeability barrier.

With the use of ultrasonic irradiation of a suspension of human erythrocyte stroma in a 10% butanol solution., Morgan and Hanahan (1966) were able to obtain a soluble lipoprotein of the stroma. The average molecular weight of the protein moiety was 163,000 and it was characterised by an N-terminal glutamic acid. The exact nature of the lipoprotein interaction is not known. Mitchell and Hanahan (1966) were also able to isolate aldolase, glyceraldehyde phosphate dehydrogenase, carbonic anhydrase, adenosine deaminase and acetyl cholinesterase from the stroma membrane. Schier (1966) working on the organisation of enzymes in the human erythrocyte membrane found a tendency for them to be orientated towards the interior of the cell.

Arrangement of Membrane Components.

Parpart and Ballentine (1952) made the distinction between loosely, weakly, and strongly bound lipids after experiments in which they treated bovine red cell "ghosts" with solvents of increasing potency. Cholesterol was found to be loosely bound and sphingomyelin strongly bound while the cephalin fraction exhibited more complex behaviour. If it is assumed that electrostatic forces hold together the protein and lipid components, while Van der Waals forces affect cohesion between the paraffin chains, the differences in behaviour of the lipid fractions can readily be understood. On the basis of these results Parpart and Ballentine proposed a structure for the erythrocyte membrane quite unlike the

Davson-Danielli model. The membrane is pictured as consisting of a continuous protein network possibly two molecules thick with lipid molecules interspersed in a three dimensional pattern. It was suggested that as well as aqueous channels or pores crossing the membrane there were water pools within the membrane.

The results of Luzzati and Stoeckenius already mentioned suggesting a non-lamellar arrangement of phospholipids agree well with the observations of Parpart and Ballentine. Further evidence suggesting that a mosaic arrangement might exist in the erythrocyte membrane came from the experiments of Dourmashkin et al. (1961) Electron micrographs of saponin treated erythrocytes stained with phosphotungstate showed a hexagonal array of pits. Prior treatment of the cells with digitonin prevented the formation of pits. As this blocking was quantitative it was suggested that the pits originally contained cholesterol. Dourmashkin found similar hexagonally arranged pits in other membranes similarly treated. However, Bangham and Horne (1962) and Glauert et al. (1962) have shown that the hexagonal patterns could be artefacts caused by the rearrangement of surface cholesterol in the presence of saponin during the preparation of sections rather than indicative of any pre-existing structure in the cell surface. Films of cholesterol alone when treated with saponin and negatively stained give similar pictures to those obtained by Dourmashkin et al. Finean and Rumsby (1963) working with "ghosts" have suggested that the hexagonal appearance

may be due to cholesterol separating from the structure in the last stages of dehydration. Betraud and Hedley (1963) have found that the fine structure of a marine protozoan shows a hexagonal array of hollow cylinders. No surfactant was used in the preparation and it was stained after mounting with potassium permanganate so that there was no separation of the lipid phase on drying. Thus there may be a hexagonal arrangement in some membranes. More recently Lucy and Glauert (1964) have described the assembly of macromolecular complexes composed of globular micelles. It is obvious that the phospholipids need not be considered as existing solely as bimolecular leaflets within membranes. The lamellar structures regarded as representing bimolecular leaflets may be formed by the association of globular micelles of lipid. Whether extensively found or not, a mosaic structure provides a more interesting structure for the description of permeability phenomena.

As well as being suitable for the study of the structure and composition of isolated membranes, the erythrocyte is useful for permeability studies. It is a free cell readily obtained and the large number available, 5×10^9 in one millilitre, ensures that the mean behaviour of a large cell population is observed. Because of its bioconcave shape it can change volume without an increase in area so that equations describing fluxes are simplified.

The erythrocyte is also simple in structure and metabolic pattern and it is relatively easy to separate glucose metabolism from its penetration. On account of the high refractive index of the contained haemoglobin, optical means can be used to follow volume changes resulting from glucose penetration or loss thus giving indirect information of glucose permeability. Direct chemical analysis is also relatively easy.

Studies on Permeability - Historical.

The cell may be considered as being surrounded by a barrier of lipid and protein. From the nature of the membrane discussed above it is not surprising that permeability studies have emphasized as did those of Hedin, the importance of lipid solubility. There is also the possibility of a substance penetrating through pores since the work of many authors including that of Ørskov and Höber supports the dependence of solute penetration on molecular size.

The lower alchols, methyl alcohol and ethyl alcohol are thus able to penetrate rapidly, but there is a marked slowing if a group of polyhydric alcohols is ascended. Hexahydric alcohols do not penetrate. Thus larger hydrophilic molecules such as sugars and amino acids which are necessary for the maintenance of the cell would be unable to penetrate unless some special mechanism existed. Penetration of such substances, especially glucose, has occupied the attention of many workers.

Research into the permeability of erythrocytes to glucose

started in 1855 with the studies of Otto, who, having compared the distribution of glucose in whole blood with that in plasma and fibrin, concluded that the red blood corpuscle was deprived of glucose. This was confirmed by Grynns (1896) and Hedin (1897) using haemolysis techniques on chicken cells and by Abderhalden. (1898). The question seemed settled until 1909 when Rona and Michaelis affirmed that the red blood corpuscle was permeable to glucose. Hollinger (1909), a contemporary worker came to the same conclusion. While many workers believed that the red cell was impermeable to glucose, others including Kozawa (1914), Masing (1914), and Ege (1919) found glucose in the erythrocyte. Masing and Kozawa using haemolysis techniques found that while the majority of mamalian erythrocytes were impermeable to glucose, those of man, monkey and dog were permeable.

Brinkmann and Van Dam (1919) found glucose in the red blood corpuscles of man and frog, but concluded that it was present only as a result of damage to the cell. The concept of damage to the red blood corpuscle was developed by Van Creveld and Brinkmann (1921) who performed analyses on plasma and cells, separated in a length of exterpated jugular vein by centrifugation.

In 1924 Falta reappraised the whole situation. He found that the glucose in the plasma varied between 71 and 124 mg. %, and that in the red cells between 40 and 100 mg. %. The results were constant whatever the mode of collecting and it was, therefore, concluded that varying results obtained previously were not due to alterations

in the membrane butto technical errors.

Most authors including Ege, Gottlieb and Rakestraw (1925), Ege and Hansen (1927) and Andreen-Svedberg (1931) seemed to agree with the conclusions of Falta save for Glassmann (1926), who upheld that there was no free glucose in the erythrocyte. For this reason it was surprising to find Olmsted (1935) maintaining that the presence of glucose was solely due to a modification of corpusclar permeability by oxalates. Olmsted's results were subjected to the criticism of Neuwirth (1936) and then Klinghoffer (1935), who repeating the experiments with the same techniques as Olmsted concluded that the addition of oxalates did not alter the proportion of glucose in the plasma relative to that in the corpuscles. He also showed that glucose equilibrated rapidly if its concentration did not exceed 2%.

It seems that one of the reasons for the variance between authors lies in the fact that they were preoccupied with a complete separation of the plasma from the corpuscles and subjected the cells to extensive washing procedures with solutions low in glucose. Another cause of error is the indifferent use of red blood corpuscles from human and other species.

As this field developed it became increasingly obvious that the process of penetration could not be considered to be one of simple diffusion. In 1897 Hedin had shown that red cells were impermeable to inositol, an isomer of glucose. In 1914 Kozawa, working with two-thirds isotonic sugar solutions, demonstrated that

various monosaccharides exhibited different rates of penetration and in 1928 Fleischman reported similar results. Ege (1919) showed that the rate of sugar penetration was affected by the concentration of the sugar solution. Bjerring (1932) confirmed this and also noted a lowering in the diffusion rate when glucose concentration was raised in an artificial system involving a collodion membrane.

The early work generally involved chemical methods or haematocrit volume changes for the estimation of sugar movement and often haemolysis. Such techniques although providing qualitative changes gave no quantitative results. In 1935 Ørskov developed a technique by which volume changes of the erythrocyte, indicative of solute movement, could be followed by recording changes in light transmission of cell suspension with a photocell and galvanometer. This method has since been developed and used extensively in subsequent work in the field of sugar permeability. The present study has involved the use of this method which will be discussed later. Very recently isotopic methods have been used. Using the photo-electric apparatus Ørskov and Bang showed that the movement of various electrolytes into the red cell did not appear to be in accordance with Ficks Law. Wilbrandt, Guensberg and Lauener seven years later published quantitative studies showing that the penetration constant for glucose calculated on the basis of Fick's Law decreased with raised glucose concentrations.

Kinetic Studies - Possible Mechanism of Glucose Transfer.

In recent years, therefore, workers have had recourse to postulating a special membrane mechanism for sugars. Apart from simple diffusion a substance may be transported across a membrane by at least four other processes and these have all to be noted when considering sugar penetration. It is not possible to treat them in detail here but they are:-

a. Entry by diffusion through organised channels or aqueous pores as for example the altered diffusion mechanism described by Faust (1960).

b. Entry by pinocytosis claimed by Barnett and Ball (1960) to be increased by insulin in adipose tissue.

c. Entry by facilitated diffusion. This could be produced in a number of ways. Those considered include a contractile protein (Goldacre 1952, Bowyer 1956) and an enzymic mechanism. Danielli suggested in 1952 that alkaline phosphatase was the enzymic contractile protein postulated by Goldacre. Rosenberg and Wilbrandt (1952) have proposed the combination of an enzyme and a carrier. Facilitated diffusion could also take place through binding to a sterochemically specific component or components extending through a pore, either temporary or permanent, in the membrane as envisaged in the polar pore hypothesis of Danielli (1954) and the polar 'creep' hypothesis of Bower and Widdas (1956)

d. Entry through active transport. This mechanism requires

both a structural relationship between the membrane and permeating species and a supply of energy. It is unlikely to be responsible for glucose entry into red cells as Morgan and Kalman (1958) have reported that no ATP is required.

The mechanism adopted as a working hypothesis by those researching in this field is that of a carrier mediated facilitated diffusion entailing association of sugars with highly specific "carrier" molecules. This carrier which may be lipid, lipoprotein or protein is by implication mobile. There is a body of evidence indicating that a limited number of highly specific sites in the membrane are involved in the transfer of many hexoses. This evidence, reviewed by Widdas in 'Recent Advances in Physiology' (1963), will not be given fully but includes evidence of substrate specificity, species differences, kinetics differing from simple diffusion, mutual inhibition by pairs of sugars or sugars and drugs which are competitive inhibitors, inhibition by mercuric compounds and other reagents such as dinitrofluorobenzene (DNFB)

The general principles of a carrier mechanism were first considered by Osterhout (1933) who discussed the possibility of transfer involving reversible combination with some cellular element present in constant but limited quantity. Shannon (1938) examining renal function reviewed the mechanism. Ussing (1952) coined the term "carrier".

Very simply the substrate is pictured as complexing with the carrier at one membrane interface, possibly by hydrogen bonding.

The complex, presumed to be relatively soluble in the membrane diffuses across the membrane and the substrate is released at the other interface. The carrier is envisaged as passing backwards and forwards across the membrane as a result of thermal agitation, whether or not it is complexed with sugar. Several other assumptions are made in considering this model which, in the light of experimental results, seem to a large extent to be justified. In formulating a simple kinetic model for the placenta, a model which was later applied to the erythrocyte (1953), Widdas assumed that the process was symmeterical, that the carrier came into rapid equilibrium with the substrate at each interface and that the transfer of the complex was the rate limiting step. Lefevre (1948) suggested that the complex dissociation was rate limiting, but this did not fit so well with the experimental observations.

Widdas applied the Langmuir adsoprtion isotherm to derive the fraction of carriers saturated (θ). This is given by

$$\theta = \frac{C}{[C + \phi]} \qquad (1)$$

where C is the hexose concentration and ϕ is the concentration of sugar which gives 50% saturation of the system i.e. the half saturation constant.

The net transfer rate will thus be proportional to the differences in the saturation fraction for the carriers at the two sides of the membrane.

$$\text{Thus: } \frac{dS}{dt} = K\left[\frac{Co}{Ci + \phi} - \frac{Ci}{Ci + \phi}\right] \qquad (2)$$

where Co and Ci are the external and internal concentrations of hexose (S) and K is a constant proportional to the total amount of carrier.

Widdas showed that approximations for equation (2) can be made in two limiting cases. When the ~~equilibrium~~ half-saturation constant Ø is large compared with the sugar concentration then the equation takes the form:

$$\frac{dS}{dt} = \frac{K}{Ø} \left[Co - Ci\right] \qquad (3)$$

Under these conditions it may be seen that the transfer rate depends directly on the difference between the sugar concentrations. The kinetics are, therefore, of the diffusion type.

When the constant Ø is small compared with the sugar concentration, the equation has the form:

$$\frac{dS}{dt} = KØ \left[\frac{1}{Ci} - \frac{1}{Co}\right] \qquad (4)$$

Here the rate depends on the difference of the reciprocals of the sugar concentrations and the kinetics are of the saturation type. Equation (4) may be rewritten as:

$$\frac{dS}{dt} = \frac{KØ}{CiCo} \left[Co - Ci\right] \qquad (5)$$

in which transfer is proportional to the concentration gradient across the membrane as appropriate to a process of diffusion. The "constant", however, depends on a term including the concentrations of sugar in the denominator and it can be shown that it would vary approximately as the inverse of the square of the outside concentration so that the following equation should hold.

$$\log K \propto -2 \log C$$

where K is the diffusion coefficient e.g. K/Ø of equation (3)

Widdas (1954a) showed that his experimental results and those of Wilbrandt et al. (1947) agreed well with this approximation.

A phenonomenon resulting from this concept of carrier transport which would be difficult to explain on any other basis is that of uphill transfer by counterflow. Widdas (1952) predicted that if a substance (X) were equilibrated on two sides of a membrane and a second substrate for the carrier system added to the outside, a temporary transfer of X against its concentration gradient would result. This prediction was tested by Park et al. (1956). Rosenberg and Wilbrandt were able to demonstrate the same effect using glucose and mannose labelled with ^{14}C so that the fluxes could be followed. (1957)

Of the two parameters in Equation (2), K is the capacity factor related to the number of carriers and their rate of movement and so depends on the character of the cell and the species in question. It will be affected by chemicals influencing the orientation and interaction of membrane constituents. On the basis of the assumptions made, a constant value for K should be found for different sugars using the same carrier within a certain membrane and Lefevre (1962) and Miller (1965) have shown this to be the case. They obtained values similar to those of Sen and Widdas (1962) in the region of 0.25 isoquantities/min at 20°C. The affinity factor Ø depends on the nature of the carrier and the sugar involved and so

might be expected to have similar values for a given sugar over a range of species. This is in fact found to be the case. A number of authors found that the half saturation for glucose only varies over the range 4 - 10 mM in human erythrocytes, rabbit erythrocytes, rat heart and frog sartorius.

Several methods have been used to determine $\emptyset g$ ($\emptyset$ for glucose) and K. Estimates of $\emptyset g$ for human erythrocytes have been made from experiments on entry of glucose into the erythrocyte using a competitive inhibitor, (Lefevre, 1954) or another sugar (Widdas, 1954). The parameters cannot be measured directly as the entrance into an ever increasing sugar concentration is constantly slowed and the maximum entry rate is not seen. Sen and Widdas (1960), however, developed a method used in the present work in which exits of glucose were followed from cells equilibrated with glucose into a saline medium of low but known glucose concentrations. The value of $\emptyset g$ obtained by the Sen and Widdas technique is, however, only about half that derived from entry experiments and this suggests that unwarranted approximations may have been made.

More sophisticated equations than the one quoted above may be derived including rate constants for all the various steps, when it becomes apparent that the parameters $\emptyset$ and K include a number of rate constants.

In considering the model described it was assumed that free and bound carriers move at the same rate. The experiments of Britton (1964) and Harris (1965) on exchange diffusion seem to indicate that

this is not the case. Levine et al. (1965), estimating the relative rates for movement of saturated and free carriers gave a figure of 2.8 and Mawe and Hempling estimated the ratio to be at least four, (1965).

The kinetic treatment of the carrier concept discussed so far allows only for the possibility of a single sugar molecule reacting with the carrier. Stein (1962) suggested that some sugars crossed the membrane in the form of dimers. He followed sugar uptake when pairs of sugars were present and found a systematic deviation of the initial swelling rate which he explained on the basis of dimerisation. Miller (1966) was unable to confirm his findings and Lefevre (1966) also could find no evidence for this hypothesis. It is known that two substrate molecules are required for the functioning of some enzymes, so it could be that two or more sugars are required to react with the carrier. Kotyk and Wilbrandt (1964, 1965) have considered the kinetics involved in a mechanism using dicomplexes. Their experimental results seem to be in accord with this hypothesis. Britton (1966) has also given consideration to a polyvalent carrier system. The possibility is an interesting one.

It must be emphasized that the existence of carriers is still hypothetical, but as the body of evidence supporting the assumption grows, so the importance of carriers in physiology grows. There is now evidence that many substances enter the erythrocyte

by carrier mediated transport - amino acids (Reiser 1961, 1962; Winter and Christensen 1964) purines, pyrimidines (Lassen et al 1961, 1962), and urea, ethylene glycol, glycerol and erythritol (Hunter et al 1965). It is possible that some compounds share the same carrier.

It seems that the facilitated glucose transfer is a fundamental property of most cell membranes. That in the red cell probably represents the simplest form of this mode of transfer. In other systems such as kidney tubule cells and luminal intestinal cells transport is linked with an accumulating system and in bacteria as first proposed by Cohen and Monod, permeases are believed to operate. Koch (1964) visualised permeases as catalysing the reaction between the carrier and the sugar.

Present Problem.

In spite of extensive studies on the problem, all tending to confirm the presence of some reactive component within the membrane, comparatively little is known of the mechanism underlying glucose transport across the membrane. Even the exact nature of the chemical grouping required for the molecule to be easily permeable is not known. Levine (1952) has reported D-glucose, D-galactose and L-arabinose ~~do not~~ readily transport into the red cell. These sugars share the same configuration at C_1, C_2, and C_3 but this does not hold for D-mannose which also penetrates readily. Lefevre and

Marshall (1958), who made an extensive study found good agreement with the suggestion that the affinity of the transport system requires the conformation of the pyranose ring to be the chair form.

The object of the present work was to obtain information as to the chemical nature of the suggested glucose carrier. It was felt that it was important to characterise the components of the membrane on which glucose transfer depends in several different ways. It was proposed to adopt three main lines of approach:- kinetic studies which would involve the use of inhibitors; inhibitor uptake studies; and biochemical extraction procedures.

The first group of studies, which were kinetic, were planned to elucidate the nature of the reactions involved and give more information which could be useful for the proposed extraction experiments. It was proposed to study chiefly the action of inhibitors following the general line of Sen and Widdas and to extend the observations of Dawson and Widdas on the effect of these inhibitors on the glucose transfer parameters and the effects of temperature on the inhibitory processes. Dawson and Widdas found that NEM inhibition as with DNFB had a high Q_{10} suggesting that the inhibitory reaction is complex, (1963).

Of special interest as inhibitors are the diphenols. As early as 1899 Von Mering had observed that phlorrhizin could inhibit sugar reabsorption in the renal tubules. Doubt was cast on assumption that its inhibitory action in the erythrocyte and heart muscle was the same as in kidney, by the observation of Wilbrandt

that the aglucone phloretin was many times more potent. Lefevre (1959) had studied the action of derivatives of phlorrhizin and allied molecules. He found that the breakdown products of phloretin, containing one or other of the two of the phenolic groups, are relatively inert, whereas some diphenols from amongst the synthetic oestrogens are as potent as phloretin. Studies of diphenols and similar compounds, therefore, appeared to be of some value.

It was also hoped that by examining a range of inhibitors it could be seenif there was one more specific for the transport mechanism.

It was hoped that the second experimental approach would indicate the possible number of sites available and provide further information for the proposed extraction and isolation experiments. Uptake of inhibitor could best be followed by using a non-competitive inhibitor. Not all of the many inhibitors citedin the literature were suitable for the proposed study. Tannic acid and n-butanol as reported by Hunter et al. (1965) were thought to act on a portion of the membrane involved with the movement of glucose carrier complexes. Mercurial compounds have been shown by Lefevre (1948) to be inhibitors, but they are relatively non-specific and inhibition can be reversed by adding excess of compounds such as cysteine. Other SH blocking agents such as Cu^{++} and alloxan are ineffective. Widdas and his co-workers (1956, 1958 and 1963) have made extensive studies with NEM and DNFB, commonly called protein reagents. DNFB which reacts with thiol groups may also

react with amino and phenolic hydroxyl groups. Bowyer and Widdas (1958) found that the progressive irreversible inhibition of DNFB proceeded more rapidly when cells were incubated with inhibitor in the presence of glucose and suggested that the action of glucose might have as its basis exposure of active groups at the cell surface. This was to some extent confirmed by the observation that the hydrogen bonding agents, urethane and guanidine, exhibited a similar effect to that of glucose. The availability of NEM and DNFB labelled with ^{14}C, therefore presented a valuable opportunity to investigate this type of inhibition.

In the third group of studies it was proposed to try to isolate red cell membrane components which may be involvedin sugar transfer or the reaction with inhibitors. Isolation of the material involved would obviously be an important step in elucidating the fundamental mechanism of transport. Although red cell stroma have been extensively studied as described earlier there have been few attempts to correlate the structure and permeability (except for the work of Van Deenen). As there is no 'in vitro' method for testing the substance, a system of stable marking must be adopted, and one object of the extraction techniques was to evaluate the relative merits of marking with various ^{14}C labelled compounds.

The work to be described was chiefly concerned with the extraction of lipids. There has been much speculation as to the chemical nature of the carrier, whether it be protein, lipo-protein or lipid in nature. The high degree of specificity involved, the efficiency

of various protein reagents and the sensitivity to metal ions are indicative of a protein being involved. The high activation energies required for formation and dissociation of complexes and the established fact of permeases in bacteria also suggest that a protein, possibly an enzyme, is required. On the face of it, however, most evidence seems also to implicate lipids. The need for mobility and the requirement of lipid solubility as stressed by general membrane permeability studies favour the involvement of lipid alone or lipid in conjunction with protein. The experiments of Reinwein reported by Park (1960) and of Hillman et al. (1965) lend support to the hypothesis of a lipid substance as a controlling factor. Lefevre et al. (1964) obtained ^{14}C glucose associated with lipids in extracts of ghosts and red blood cells obtained through the use of hot ethanol-ether. One significant fact was that if the red cells were incubated with DNFB before adding glucose and extracting the amount of glucose obtained was reduced by two thirds.

It was proposed to attempt the marking of lipids with various radioactive labelled inhibitors and also ^{14}C glucose. This last step would obviously be expected to present difficulties owing to the temporary nature of the complex.

CHAPTER 2.

Materials.

All chemical reagents were analar grade and solvents redistilled before use.

Fresh blood was obtained by venipuncture and collected in a heparinised tube.

Time expired blood from the North London Blood Transfusion Centre, Deansbrook Road, Edgware.

Sheep blood - defibrinated from Burroughs Wellcome.

Silicic acid for chromatography - 100 mesh; Sigma.

Hyflo-Supercel; Hopkins and Williams.

DEAE cellulose (DE 50); Whatman.

Silica Gel G; Merck; Anderman & Co. Ltd., London.

Silica Gel H; Merck: Anderman & Co. Ltd., London.

Spray reagents: Merck: Anderman & Co. Ltd., London.

1. Ninhydrin.
2. Phosphomolybdic acid.
3. Analine phthalate.

^{14}C-N. ethyl maleimide specific activity 10 µc/ml.; Schwarz BioResearch Inc. U.S.A.

^{14}C labelled compounds from the Radiochemical Centre, Amersham, Bucks.

1. D-Glucose-C14 - U.- specific activity 2:4 mc/mM.
2. N-ethyl (maleimide - 2, 3 - C14) specific activity 15.89 mc/mM.
3. 1 - fluro - 2, 4 - dinitrobenzene - C14 - specific activity 50 µC/mg
4. Diethyl stilboestrol (monoethyl - 1 - C14) specific activity 28 mc/mM - disolved in benzene.

Standard Lipids.

Cardiolipin Antigen; Burroughs Wellcome, London.

Cardiolipin in absolute alcohol; Nutritional Biochemical Co. Cleveland, Ohio.

Cerebrosides; Koch-Light Laboratories, Colnbrook.

Synthetic cephalin; Nutritional Biochemical Co.

Phosphatidyl ethanolamine; Koch-Light Laboratories

DL-Alpha Lecithin (synthetic); Nutritional Biochemical Co.

Lysolecithin (cadmium chloride complex) Koch-light Laboratories.

Phosphatidyl inositol (ex beef brain, Folch fraction 1); Koch-Light Laboratories.

Sphingomyelin; Koch-Light Laboratories.

CHAPTER 3.

General Methods.

Kinetic Studies.

As was stated in the introduction, for studies in which the net flux of a sugar was followed, a photoelectric apparatus was employed. Following the work of Ørskov, Widdas developed a stable sensitive apparatus which could follow accurately small changes of cell volume, resulting from movement of solute and accompanying water. The apparatus has been described by Widdas (1953a, 1954) It employs a chopped double light beam system with an a.c. amplifier and a phase sensitive rectifier. The output operates a pen recorder and over a limited range of volume changes (maximal 23%). The excursion of the pen recorder is proportional to the volume change. The cell suspension is contained in a glass cuvette.

The advantages of using this method are that deparatures of the cell volume from the physiological value can be quite small and the cells can be suspended in physiological fluid (which is not possible with the haemolysis technique).

Solutions.

Stock Solutions. A-Phosphate Buffer - 15.6 g NaH_2PO_4 were dissolved in 450 ml. de-ionised distilled water and 35 ml of 2 N-NaOH added. Additional amounts of NaOH were added until a pH of 7.4 was achieved and the solution was made up to 500 ml., (dilution changes of pH were found to be negligible).

B. - Sodium Chloride - A 10% solution was prepared by dissolving 50 g in 500 ml.

A solution of milliosmolarity 342 (isotonic with human plasma)

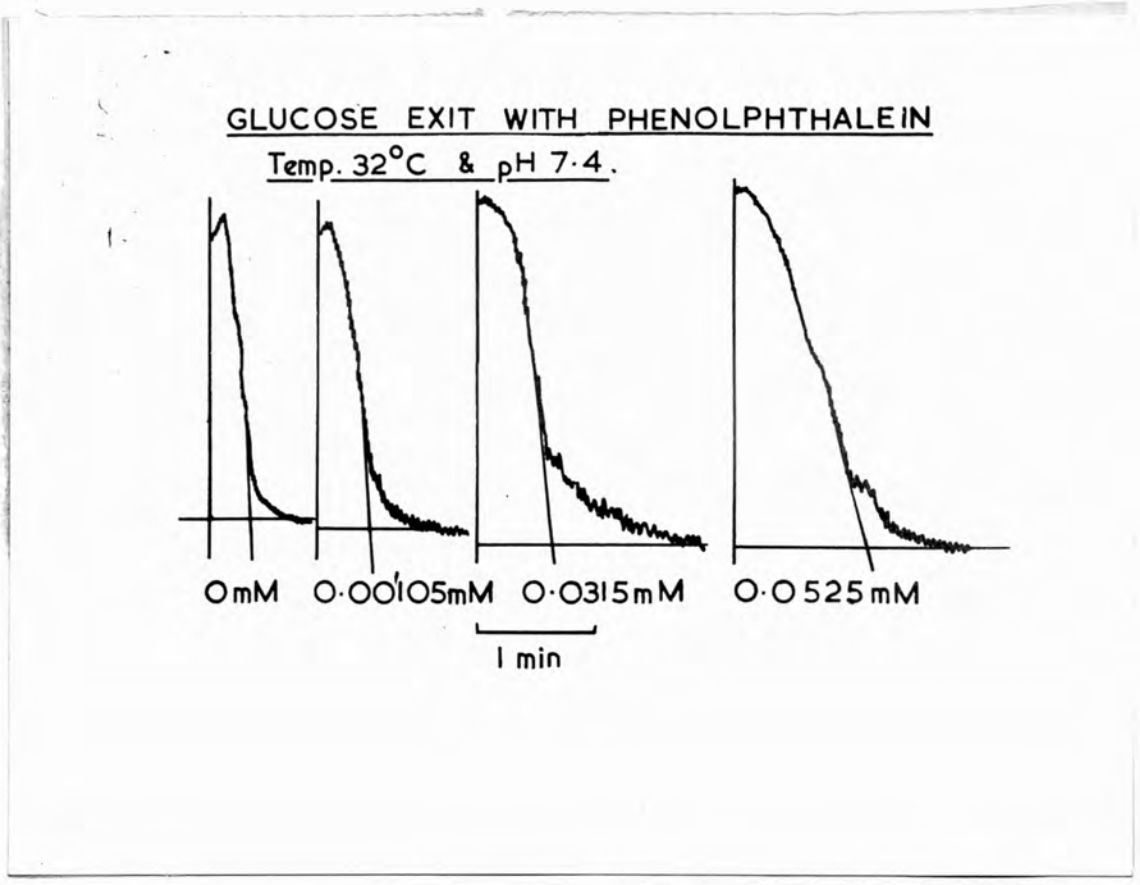


Fig. 3.1. Tracings of a series of records from the photoelectric apparatus during exit experiments. Cells equilibrated in 76 mM glucose were losing glucose into media containing 0.8 mM glucose and phenolphthalein in the concentrations shown - ranging from 0 mM to 0.0525 mM. Note that the linear character over the first two thirds of the exit was maintained throughout. This linear portion was produced to cut the base lines as shown and the time from injection of the cells to this point measured in each case.

was prepared for use by diluting 25 ml. of A and 20 ml. of B to 250 ml. with distilled de-ionised water.

A fresh 30% glucose solution was prepared daily. This would be diluted by a factor of 20 before introduction into the cuvette for exit experiments (Sen and Widdas 1962a).

All solutions were filtered before use (Whatman no. 50) as small dust or fibre particles disturb light transmission.

Procedure for following Glucose Exits.

The general procedure was as follows:- Red cells, washed three times in phosphate buffer, were suspended in a medium containing 76 mM glucose, the suspension being prepared with 0.2 ml. packed cells, 10.3 ml. buffered saline, and 0.5 ml 30% glucose solution. The cells were incubated for thirty minutes at 37°C and then packed by centrifugation for four minutes. Three cu mm of packed cells were taken up in a blood pipette and transferred to a small pyrex tube containing 0.2 ml. of the supernatant recovered after spinning the cells. In future, as far as experiments using this technique are concerned, the term "supernatant" will be used to denote solution in which the cells were equilibrated with glucose. The cells were thoroughly mixed with the supernatant by drawing up the solution and discharging it before pipetting it into the cuvette. The cuvette contained 21 ml. of saline buffer which was either glucose free or contained low concentrations of glucose or various

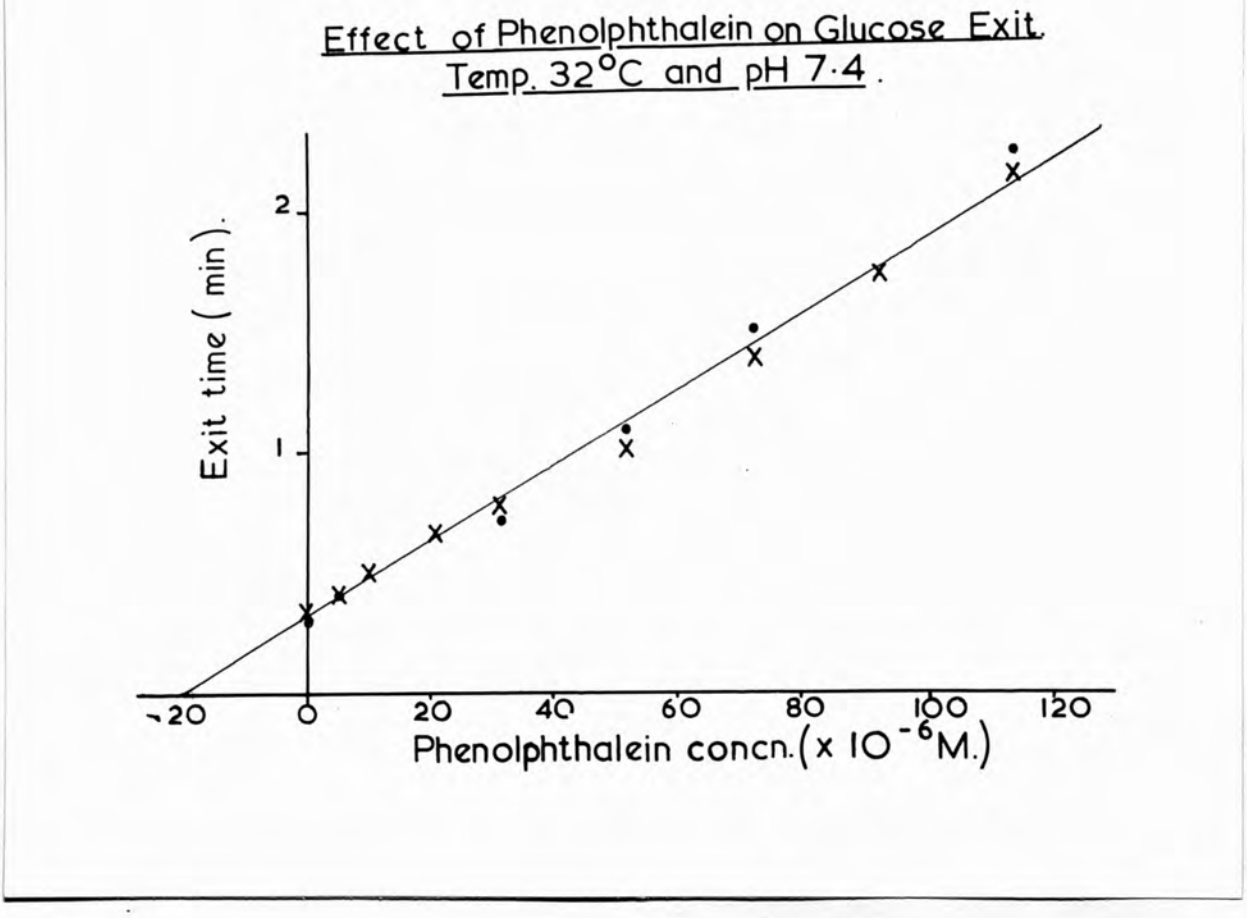


Fig. 3.2. Exit times at pH 7.4 and temperature 37°C obtained as described in the text, plotted against phenolphthalein concentration in the outside meium.

The line gives two intercepts, that on the ordinate (t_o) represents the time which would have been taken før exit into a medium free of phenolphthalein and that on the abscissa represents the concentration of phenolphthalein in which the exit time would be twice t_o. This last value equals the half saturation constant.

inhibitors. When the cell suspension was added to the cuvette a rapid initial adjustment was made of the shutter controlling the reference beam to compensate for variations in the density of the cell suspension. The cells first swelled osmotically and the recording pen moved in the direction of decreased light transmission. Then as the glucose came out of the cells they shrank and the pen moved in the direction of increased light transmission. The majority of the volume changes traced were approximately linear, as exemplified by fig. 3.1. Exits into varying media were performed at least in duplicate.

Calculation of Results.

The linearity of the trace was taken as evidence that the efflux process was nearly saturated. By producing the linear portion of the trace to the base line, a time for the whole exit, if it had proceeded at the fully saturated rate could be obtained. Fig. 3.1. illustrates the point that this time increases when the outside medium contains increasing quantities of sugars or inhibitors of sugar transfer. The measured times were plotted against the concentration of added solution in the cuvette as shown in fig 3.2. (after Sen and Widdas 1960 a). The intercept on the ordinate gives the minimum time for exit and the concentration of substrate required to double this time is given by the intercept on the abscissa. This value represents the apparent half saturation constant for the material in question and was obtained in general

by calculating the regression of x on y. The 95% confidence limits for a single estimate are of the order of ± 15%. When three or more observations were made this reduced to ± 5%. Mean values only are given in the text.

Preparation of Tissue and Extraction of Lipids.

Preparation of Erythrocytes.

Time expired cells from a "Blood bank" were suspended in approximately five volumes of saline-buffered at pH 7.4 spun for twenty minutes at 4,000 r.p.m. and the supernatant removed. The washing was repeated twice. The cells were discarded if any degree of haemolysis was observed. In experiments using whole red cells the haemoglobin was found to take up a great quantity of the inhibitor labelling and its presence generally increased the difficulty of lipid separation so experiments were performed using red cell ghosts.

Preparation of Erythrocyte Stroma.

The red cells were washed as described above. In initial experiments ghosts were prepared by first haemolysing with distilled H_2O containing 50 mg EDTA in 100 ml. and spinning down at 20,000 r.p.m. for thirty minutes. The ghosts were then washed six times in 0.15% saline with EDTA buffered at pH 7.4. Spinning at 20,000 r.p.m. for twenty minutes was sufficient to separate the cells from the saline which was carefully removed

with a Pasteur pipette, a strong light being used behind the tube so that the boundary could be more clearly seen. The method, essentially similar to that of Dodge, et al. (1963) was quite effective, 0.3% of haemoglobin remaining.

In the majority of experiments the ghosts were prepared by Miss D.A. Harris who followed the method of Post, et al. (1960) with further modifications of Hokin and Hokin (1964). The cells were haemolysed and then centrifuged. The concentration of haemoglobin was further reduced by washing with 5×10^{-3} M tris-chloride buffered at pH 7.4. The resulting ghosts contained less than 0.1% haemoglobin. Phase contrast microscope photographs taken show that the cell structure was retained intact. Also the independent work of Hanahan and Finean using the diffraction pattern of ghosts has shown that the lipid protein cellular structure was retained. These workers also investigated which enzymes were removed in the process of preparing ghosts.

Extraction of Lipids.

The erythrocytes or erythrocyte ghosts were incubated with the radioactive inhibitor or ^{14}C-glucose for the required length of time and then extracted with ten volumes of one of several lipid solvents. The incubation with ^{14}C-glucose was carried out at 0°C. for one minute to reduce any metabolic incorporation. The extraction was also carried out at 0°C. as at the lower temperature the dissociation of the complex is reduced (Sen and Widdas, 1962a)

There are many problems involved in lipid extraction, but these have largely been overcome in the last ten years. Several reviews on the subject have been written, e.g. Levine (1955). Entermann and Sperry (1955) and Marinetti (1962). As the erythrocyte membrane consists of lipid with a protein covering it is necessary first to break the bonds between lipids and protein before the lipids can be taken up in a fat solvent. This may be achieved with any of the low membered alcohols which in standard procedure are combined with a fat solvent such as chloroform. A widely used solvent mixture is chloroform:methanol.

Total lipid extracts usually contain free amino acids, peptides, sugars and other hydrophilic natural products which are carried into the extract through the action of lecithin and other solubilizers. For chloroform:methanol extracts the Folch washing method is the most convenient way of purifying and generally it seems the most effective method is to subject the preparation to chromatography.

As the object of the experiments was to determine whether glucose or an inhibitor could be obtained complexed with a membrane constituent, certain stringent requirements were put on the extraction method to be used. The introduction of any procedure involving an aqueous solvent was considered undesirable as any glucose - lipid complex would most likely be very labile and probably dissociate at the interphase. Lengthy procedures were also to be avoided to prevent chemical changes during treatment or formation of complexes not present when the membrane was extracted. Since the aim at this stage was

simply separation rather than identification of any complex, the methods were kept as brief and as simple as possible and it was hoped that a degree of purification would be obtained during the initial separation on silicic acid columns.

Lipids are liable to become exidized and undergo other alterations during handling. Therefore extensive care was used during extraction and all other procedures. Purified solvents were used and wherever possible procedures were carried out under an atmosphere of nitrogen. Heat was only used when absolutely necessary. The lipids were stored in the cold at 4°C. in tightly stoppered flasks or tubes.

Pilot experiments were run in the laboratory to test the relative merits of a number of solvent systems including chloroform: methanol 5:2, petroleum spirit, ether and ethyl alcohol used successively and n-butanol. On the whole butanol was found to be the most suitable solvent giving a good yield of lipid and a high recovery of radioavtivity. A further advantage of butanol is the slight miscibility within water so that the small quantities of water present in the cell preparation did not separate out to any great extent.

The procedure adopted for incubation with ^{14}C-labelled glucose and butanol extraction was as follows: 30 to 40 ml. of ghosts were mixed with 5 to 10 ml. of saline containing readioactive glucose and after one minute added to 10 vol of the solvent which was chilled and through which nitrogen had been bubbled. The mixture was constantly stirred during extraction to prevent clumping. The cells

were then left at 4°C overnight for complete extraction. The relatively clear supernatant was decanted off, filtered through a Buchner funnel and evaporated in portions of 50 ml to small bulk under a reduced pressure of nitrogen at 40°C . The concentrated fractions were then pooled and taken to near dryness at 32°C.

The residue after extraction, consisting chiefly of haemoglobin and membrane protein, was found to contain ^{14}C-glucose so that a further extraction was attempted using acid butanol - 100 ml. butanol:0.25 ml. concentrated hydrochloric acid. It was hoped this would break any remaining lipoprotein links. The supernatant was again decanted and evaporated to near dryness as described above.

In those experiments in which chloroform:methanol was used the red cells or ghosts preparation was poured slowly into four volumes of methanol and ten minutes after, eight volumes of chloroform were added with constant stirring. The mixture was allowed to stand overnight. The aqueous layer was removed, the chloroform layer filtered and evaporated down at 37°C under reduced pressure in a stream of nitrogen. Ethyl alcohol was added from time to time to prevent frothing. A similar extraction procedure was also followed using isopropanol:chloroform 7:11 v/v. Rose and Oklander, (1965) after extracting human erythrocytes with a number of different solvent systems

found this to be the most satisfactory.

A series of experiments were also carried out using ethyl acetate. It was felt that this might be a useful solvent as it formed a constant boiling mixture containing a low proportion of water. The ghosts were shaken with the solvent for fifteen minutes, left for forty-five minutes and the supernatant filtered off using a trap. The residue was extracted twice with a volume of chloroform. The mixture separated into two layers with a layer of material at the interphase. This material was extracted with acidified chloroform methanol.

After extraction the material was taken up usually in chloroform:methanol 9:1 for column separation.

Separation and Analysis of Lipids by Chromatographic Techniques.

Several reviews have been published discussing theoretical principles of chromatography, Williams (1954), Smith (1960), Stahl (1962) and Randerath (1963). Silicic acid column chromatography and thin layer chromatography are essentially a combination of adsorption and partition chromatography. Polar compounds are bound by electrostatic forces to the crystal lattice of the adsorbent which is an oxide previously heated to remove the surface film of water. Lipids without polar groups are not retained. The materials are eluted according to the polarity of the solvent used and their solubility in it. In adsorption chromatography the preparation of the solid phase is of great importance.

Large amounts of material may be separated by column chromatography,

but unfortunately the separation is in general incomplete (see Marinetti 1962), so that it is necessary to use another method which was chosen to be thin layer chromatography.

Trappe (1940) was the first to use silicic acid columns for the separation of lipids eluting them with chloroform and then methanol. This method has since been modified and improved by Mckibben and Tayler (1952), Lea et al. (1955) and Hanahan, Dittmer and Warashina (1957). In 1960 Hanahan et al., extended their investigations to a number of tissues including red cells. The procedure adopted in this investigation was essentially similar to that described by these authors.

Silicic Acid Column Chromatography.

Apparatus - The columns were 1-2 cm in diameter and 30 cm long. The larger ones were fitted with sintered glass discs at the bottom and the smaller had drawn out tips and were fitted with glass wool plugs. As organic solvents were used all the glass apparatus was without taps. Gas pressure was applied through an inlet at the top of the column. Fractions of 5 or 10 ml were collected using a siphon operated collector.

Procedure - The silicic acid was washed with chloroform:methanol 9:1 and dried overnight at 110°C or by careful washing with acetone and ether. Sufficient silicic acid was used to give 10 mg lipid (dry weight) per gram silicic acid: 5, 10 or 12.5 g silicic acid were generally used and a suitable column selected to give a height

diameter ratio of the order of 10. Two parts of silicic acid were mixed with one part of Hyflo-supercel to speed elution and the mixture was washed with chloroform:methanol 2:1 on a Buchner funnel. A slurry was prepared in chloroform:methanol 9:1, poured into the column and allowed to settle under gravity. The walls were washed down and the column packed down under a nitrogen pressure of 2lb./sq. inch so that a flow rate of 1 ml./minute was obtained with the smaller column and 2 to 3 ml./minute with the larger. The column was never allowed to run dry. A column of 5 gm of silicic acid, of diameter 1 inch and length 16 cm was found to have a dead space of 5 ml. so that the fractions collected were equal in volume to the dead space which may be considered to be rather high but a satisfactory separation was obtained.

The solvent was allowed to run until its level was near the top of the silicic acid then a 10% solution of the lipid in chloroform:methanol 9:1 was pipetted on to the top of the column. The lipids were eluted with chloroform:methanol 9:1, 4:1, 3:2, and 1:4, 10 ml. of each solvent mixture being used per gram silicic acid. In some experiments elution with chloroform preceeded the chloroform:methanol 9:1, and after the other mixtures had been used elution was completed with methanol. Quantities of the eluent in each fraction were taken for radioactive counts and for phosphorus estimations, so that the course of elution of glucose labelling and of phosphorus containing material could

be followed. Those fractions constituting a single peak were pooled and used for other studies; these were primarily thin layer chromatography and rapid analysis by infra-red spectroscopy. In early experiments the pooled fractions were brought to dryness and weighed, but unfortunately the results were not reliable as silicic acid was sometimes found to be present, especially in the later fractions.

Radioactive Measurements - These were carried out in duplicate, 0.2 ml. samples being pipetted onto aluminum planchets. They were dried under a lamp to give a layer of lipid infinitely thin and counted using a Geiger Muller Counter for 10 minutes or until the number of counts exceeded one hundred.

Phosphorus Estimation - The phosphorus content of the samples, containing in the region of 0.5 to 20 µg phosphorus, was estimated by the method of Taussky and Shorr (1953) as used by Rathbone (1962). To evaporate off the solvent 0.8 ml. of 10 ml. samples were gently heated. Then they were thoroughtly digested with 0.1 ml. of an acid mixture containing 10N suplhuric acid and 70% perchloric acid (1:1; v/v) on a sand bath at 120°C for fifteen to twenty hours. At the end of this time any carbon remaining was oxidised with a few drops hydrogen peroxide. The tubes were allowed to cool a little, 1.5 ml. distilled watter added to convert any pyrophosphate to phosphate and then 1.5 ml. developing reagent were added to each tube which was shaken and allowed to stand for ten minutes after which time a blue colour developed which was read at 690 mµ on the Optika

spectrophotometer against a reagent blank. Quartz cells were used with a light path of 1 cm.

The developing reagent was prepared as follows; from a stock solution of ammonium molybdate containing 50g ammonium molybdate in 400cc 10N sulphuric acid and made up to 500 ml. with distilled water, 10 ml. was taken and diluted to about 70 ml. with water. Ferrous sulphate 500 g were added and the whole made up to 100 ml. and shaken. Samples of 0.05, 0.10 and 0.15 ml. of a stock solution of phosphate containing 0.5853 g potassium dihydrogen phosphate per litre. (133.3 µg P/ml.) were mixed with the reagents and values obtained used to construct a standard curve from which the phosphorus content of each fraction eluted could be calculated. An estimate of the percentage content of phospholipid was obtained by multiplying the percentage of lipid phosphorus by 25 (Davenport 1963).

Preparation and Elution of DEAE Cellulose Column.

Attempts were made to further separate lipids eluted from silicic acid columns on DEAE-cellulose columns in the manner suggested by Rouser et al (1963). The impurities present were removed as follows. A quantity of DEAE-cellulose was washed with methanol on a pad of filter paper on a sintered glass funnel. This was followed by successive washings with 500 ml. N-HCL, distilled water, 500 ml. N-KOH, distilled water and finally methanol. The DEAE was dried under very gentle suction, squeezed gently between filter papers and dried over KOH in a vacuum desiccator.

One gram DEAE was suspended in glacial acetic acid, packed

in a small column under low nitrogen pressure and washed with three volumes of acetic acid and six volumes of methanol. The pH of the eluent was checked before further washing with chloroform and chloroform:methanol 9:1. At this stage the lipid material was introduced onto the column and eluted with the following sequence of solvents-20 ml. chloroform 9:1, 50 ml. chloroform:methanol 7:3, 20 ml. methanol; 20 ml. chloroform:acetic acid 3:1; 30 ml. chloroform:acetic acid 3:1 plus 0.005 M ammonium acetate; methanol wash (6 volumes); 20 ml. glacial acetic acid; 35 ml. chloroform: methanol: concentrated aqueous ammonia 28:70:0.35. Of the 5 ml. fractions collected 0.2 ml. were taken for radioactive counts.

Thin Layer Chromatography.

Thin layer experiments were performed essentially as described by Truter (1963), Randerath (1963), Bobbit (1963) and Stahl (1965).

Preparation of Plates - Glass plates 20 x 20 cm or 10 x 20 cm were coated to a depth of 250 μ (or 500 μ in cases where more material was to be separated) with adsorbent applied in a suspension of 30 g in 60 ml. distilled water, using a Desaga spreader. The adsorbent used was silica gel G nach Stahl (Merck) although successful separation was achieved with silica gel H which has the advantage that the slurry may be prepared in advance. The plates were allowed to dry at room temperature for ten minutes and then at 110°C for forty-five minutes in a vertical position. The position is important since only the top plate dries quickly in a rack in the horizontal

position, the other plates requiring two hours to be activated. The plates were stored for up to ten days in a desiccator over calcium chloride and cobalt chloride or blue silica.

Application of Lipid Samples - Standard lipids were applied in a 0.1% solution and samples in a 1% solution of chloroform:methanol 4:1 in spots containing 10 to 20 µg, or bands up to 2 cm broad and 15 mm from the lower edge. Unfortunately when samples low in radioactive counts were separated, the plate had to be a little overloaded with material to allow the radioactivity to be detected. Samples were spotted onto the plates under a stream of nitrogen with a blood pipette or, more conveniently with a 10 µl. Hamilton Syringe. Application under nitrogen was also carried out [Cruess and Seguin (1965).]

Development of Lipid Material - Many solvents and solvent mixtures were tried, attention being focused on those containing varying proportions of chloroform and methanol. An elutropic series is available (Randerath 1962) which gives some indication as to which solvents are likely to be the most useful, but that found to give the most satisfactory lipid separation was chloroform: methanol:water 80:30:3, a modification of the developing solvent used by Vogel (1962).

The chromatographic chambers were prepared one hour before insertion of the plate. They were filled to a depth of 1 cm and lined with filter paper soaked in the developing solvent. Tank saturation is necessary for the solvent front to run evenly. Development was carried out for 10 cm which took twenty five minutes

for the solvent given above. There is good evidence that no oxidation takes place during development (Mahadevan - cited by Mangold 1965) so that no particular precautions were taken.

Small Scale Chromatography - In order to test solvent mixtures small scale chromatography was carried out on microscope slides. Six slides were arranged on a perspex tray, the outer two having a layer of metal foil beneath them the required thickness of the layer. The silicic acid slurry was poured onto the plates and spread out and the plates activated in the usual way on a slide holder. The slides were developed in small screw top jars.

Detection and Identification of Lipids - The methods were essentially those of Skidmore and Enteman (1962) and Skipski et al. (1962) and Nichols (1964)

a.) Iodine - Lipids were first visualised by placing the plate in a sealed tank containing iodine crystals when lipids were seen as yellow to brown on a light yellow or white background. The colour quickly faded so that other tests could be applied.

b.) Rhodamine - Other universal phospholipid reagents used were rhodamine B, (0.2% in distilled water) and rhodamine-6-G (0.5% w/v in ethyl alchol). Spots were visible under an ultra violet lamp when acidic phospholipids appeared blue and others yellow.

c.) Stain for Phosphate- A blue colour was given by phosphate containing material when sprayed with a solution made up as follows:-

500 mg ammonium molybdate dissolved in 40 ml. distilled water to which 1.5 ml. perchloric acid, 1.5 ml. concentrated HCl was added

and finally the volume made up to 50 ml. with acetone.

d.) Ninhydrin - Phospholipids containing free amino acid groups were revealed as red violet spots after spraying with ninhydrin. Ninhydrin spray (Merck) was used or a modified ninhydrin reagent was prepared.

Solution I-50 ml. 0.2% anhydrous-ethanolic ninhydrin solution
10 ml. glacial acetic acid
2 ml. 2,4,6, collidine

Solution II-1% $Cu(NO_3)_2 \cdot 3H_2O$ in anhydrous ethanol

Fifty parts of I were mixed with three parts of II before use.

e.) Dragendorf Reagent - Plates sprayed with this reagent showed free choline as purple spots and choline containing substances as orange spots. It was prepared as follows:-

Solution I 1.7 g $BiNO_3 \cdot 5H_2O$ in 100 ml. 20% v/v acetic acid.

Solution II 40 g KCL in 100 ml. distilled water.

Four ml. of solution I and 1 ml. of solution II were used in 20 ml. distilled water.

f.) Ammonical Silver Nitrate - This was used to determine sugar molecules. The plate is first sprayed with:

Solution I 0.1 vol saturated $AgNO_3$ in water
20 or 100 vol acetone

and then with:

Solution II NaOH 0.5% in ethanol

Spraying with 2N ammonia bleaches out the background.

For general purposes only a, b, c and d were used in this

order, b sometimes being omitted.

The spots on the chromatograms were also identified according to position and with respect to reference lipids. The pure lipids used were those listed in Chapter II.

Recording of Results - The positions of spots could be marked on the plate using a dissecting needle and the chromatogram traced or photographed. Preserving the layer with a plastic material was not found to be very satisfactory.

Detection of Radiation - The position of radioactive samples was determined by placing the chromatogram on a trolley moving under a thin window Geiger counter. Pulses were picked up on a Ecko Ratemeter and fed to a pen recorder in which paper moved the same rate as the trolley

Recovery of Lipids from Chromatograms - Chromatograms were partially dried under an atmosphere of nitrogen, scanned and in some cases exposed to iodine vapour for a minute or so. The position of the material required was then marked. In the case of the material labelled with dinitrophenol, the yellow compounds were readily visible. The silica within the marked area was scraped free from the glass and sucked into a glass aspirator. Seventeen millitres chloroform:methanol 4:1 or 1:1 was injected into the aspirator and the lipids eluted under low nitrogen pressure. The elution was repeated twice. The apparatus is described by Goldrick and Hirsch (1964). Sometimes a portion of lipid was left on the

plate for treatment with various reagents.

Chromatography of Amino Acids - Some of the material obtained from silicic acid chromatography was hydrolysed with 5 N HCl for sixteen hours at 110°C and examined for amino acid content and for DNP derivatives when DNFB had been used.

Separation of amino acids in two dimensions was based on the method of Fahmy et al. (1961). Preparation of plates and developmental procedures were similar to those for lipid chromatography, the solvent systems being

First direction: Chloroform:methanol:17% ammonium hydroxide (2:2:1 v/v)

Second direction: Phenol:water (75:25 w/w)

The plates were dried at 110°C for ten minutes after development in each direction and the amino acid spots revealed by spraying with ninhydrin. Amino acids were identified by comparison with a plate of standard amino acids which was run and the diagrams of Fahmy et al. (1961)

Development in one direction was carried out using either of the solvent systems alone.

Chromatography of DNP Derivatives - Separation of DNP derivatives, chiefly DNP amino acids, was carried out on plates prepared as described above. Several solvent systems were used. Development in one direction was carried out using chloroform: tertiary amyl alchol:acetic acid 70:30:3 or benzene:pyridine:acetic acid 80:20:2. Two dimensional chromatography was performed using

one of two solvent sequences. The first involved the use of those solvents already mentioned in the order given. The first solvent was removed by passing a current of air over the plate for ten minutes, heating it at 60°C for ten minutes and allowing it to cool for ten to fifteen minutes. For the second sequence a mixture of toluene:pyridine:ethylene chlorohydrin : 0.8N ammonium Hydroxide 100:30:60:60 was prepared. A plate was equilibrated overnight with the lower phase in a solvent tank and then run in the upper phase. The solvent was evaporated off and the plate run in the second direction in chloroform:tertiary amyl alcohol:acetic acid 70:30:3. The spots were viewed directly or under ultra violet light and identified by comparing their relative positions in the various solvent systems used with those of standard DNP amino acids which had been run, and Rf values quoted in Randerath (1963)

Chromatography of Sugars - Chromatography of sugars was carried out on plates prepared from a slurry of 30 g silica gel and 60 ml. 0.1N boric acid. The plates were developed in butanol: acetone:water 40:50:10, sprayed with aniline phthalate reagent (Merck) and heated when hexoses appeared as brown spots.

Infra-red Spectroscopy.

Many of the samples used for thin layer chromatography were also examined by Miss J.C. Remfry using the infra-red spectrophotometer.

Infra-red spectroscopy represents an ideal way of examining lipids after very little treatment. The pattern obtained for each material are very specific and have been called "finger prints".

Schwarz (1960) described how tissue lipids in a cell extract may be identified by the use of a library of adsorption curves of pure known compounds.

The instrument used was a Perkin Elmer model 337 grating spectrophotometer, double-beam, scanning the range 4,000 cm^{-1} - 400 cm^{-1} (2.5 micron to 25 micron wavelength). Cells with windows of potassium bromide were used. The most satisfactory solvents were found to be carbon tetrachloride in the range of 2.5 to 7.5u and carbon disulfide in the range 7.5 to 26u. Samples could be recovered after this treatment and could be used for further observation including thin layer chromatography. Substances not easily soluble in the solvents mentioned were studied as mulls when, of course, they could not be recovered. Unfortunately, quantities of material recovered were, on the whole, insufficient for significant results to be obtained with this method.

Results of Kinetic Studies.

CHAPTER 4.

Studies with competitive inhibitors of glucose transfer.

Studies on Competitive Inhibitors of Glucose Transfer.

As was pointed out in the introduction, several studies (Lefevre 1959; Wilbrandt 1959: Sen and Widdas (1962) have been made on the action of competitive inhibitors, many of which are diphenols. The purpose of the present studies was to extend observations on substances known to inhibit, namely phloretin and stilboestrol and to examine others which from their structures seemed potential inhibitors. It was hoped that this would shed more light on the molecular mechanism of transfer. This study was also of interest since these substances might be used to label membrane material.

Solutions.

The compounds used were very nearly insoluble in water, so they were first taken up in the minimum volume of absolute alcohol and subsequently diluted in the medium to give an alcohol concentration of usually less than 0.5% and never more than 1%. A series of control experiments demonstrated that this quantity of alcohol did not affect glucose exits and there appeared no reason for it to alter the potency of the inhibitor.

Phenolphthalein - 10 mg of phenolphthalein were dissolved in 25 ml. absolute alcohol and made up to 100 ml. with buffered saline. By adjusting the $_{p}H$ of the solution to 8.5 so that the characteristic pink colour was produced, the concentration of the solution could be determined using a spectrophotometer set to read at 555 mμ. It was found that 24 hours after the solution had been prepared its

activity was down to one quarter of that initially. Therefore, a fresh solution was prepared before each series of readings. From the solution prepared a quantity was added to the cuvette to give the desired final concentration.

Phloretin - The phloretin was prepared from phlorrhizin by the method of Bach (1939) as used by Sen and Widdas, 1962). Two gm phlorrhizin were suspended in 20 ml. of distilled water to which 10 ml. 20% sulphuric acid were added. The mixture was heated for 90 min on a boiling water bath. The precipitate was thoroughly washed with distilled water and recrystallised from absolute alcohol. A stock solution was prepared by dissolving 25 mg in 2.5 ml. absolute alcohol. A dilution of 1:50,000 was made with buffer, final dilution being achieved in the cuvette.

Stilboestrol - A 1:50,000 dilution was used as for phloretin.

Thyroxine - A solution was prepared in saline, 25 mg in 25 ml. to give a concentration of 10^{-5}M when 1 ml. was used in the cuvette.

Results.

In all the experiments the cells were equilibrated with 76 mM glucose at 37°C and $_{p}$H 7.4.

Phenolphthalein - It was found that if cells were injected into a solution containing 1 or 2 ml. phenolphthalein, the exit time was increased. The exit was rapid at first and then slowed down so that it was necessary first to resuspend the cells in the same concentration of inhibitor as present in the cuvette.

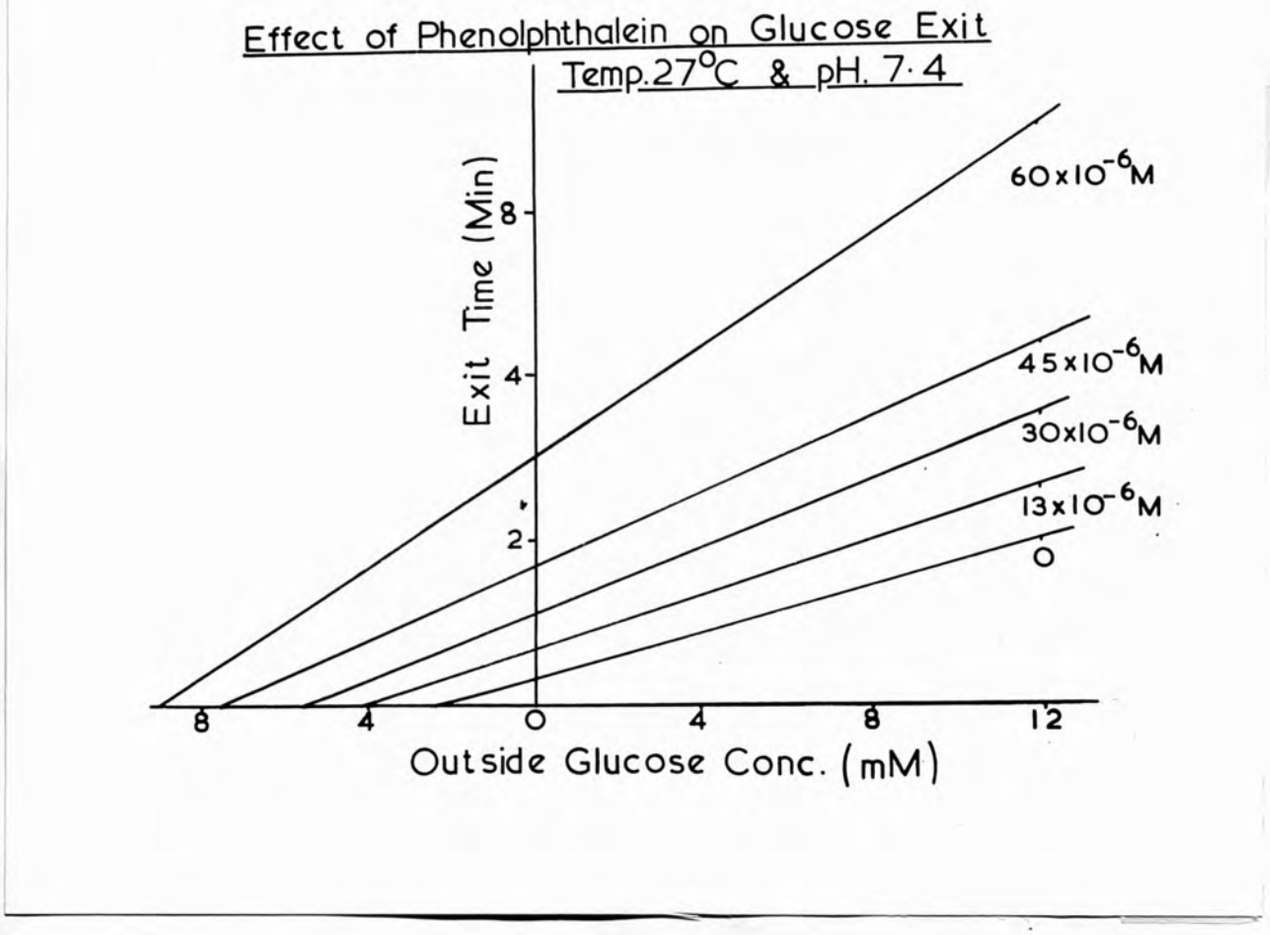


Fig. 4.1. Five regression lines showing glucose 'exit' times from cells equilibrated in 76 mM glucose, effluxing into various buffered glucose saline media in the presence of inhibitor concentrations 0 - 60 x 10^6M. Experiments at 27°C and pH 7.4

This was done by adding the desired quantity of inhibitor containing 76 mM glucose to the supernatant which was used to resuspend the cells. The exit curves then showed the same characteristics as in the absence of inhibitor, but the non-linear part was more prolonged as has previously been seen in fig 3.1. - a typical set of results.

First, exits into 0.8 mM glucose solution were followed in the presence of different inhibitor concentrations Fig 3.2. shows a plot of exit times against phenolphthalein concentration. As noted in Chapter 3 the concentration which doubles the exit time numerically is given by the intercept on the abscissa, and this concentration may be taken as the half saturation of the transfer system, for this particular inhibitor. The value obtained in this instance was 12.75×10^{-6} M at 27°C.

Second, to see whether phenolphthalein is a competitive inhibitor, glucose exits into various low glucose concentrations were followed at constant inhibitor concentrations. Thus the half saturation constant for glucose could be determined. In the presence of a competitive inhibitor the apparent half saturation constant is increased: this is found for phenolphthalein as seen in fig 4.1. where exit times are plotted against the outside glucose concentration. The individual points have been omitted for the sake of clarity, the graph being constructed from the regression lines calculated from the mean of four experiments.

The half saturation constant, about 2.5 mM in uninhibited cells at this temperature (27°C) is progressively increased in experiments with phenolphthalein in the range 1.5 to 6.0 x 10^{-6} M. The increases in values of half saturation and exit times in glucose free medium are not exactly proportional to the increase in inhibitor concentrations.

The effect of $_{p}H$ and temperature on the concentration of phenolphthalein giving 50% inhibition was also examined. The $_{p}H$ was varied over the range 6.0 to 8.4. Sen (1960) observed that the range of $_{p}H$ could not be extended below 5.3 or above 8.4 if haemolysis was to be avoided and a constant pH maintained. The pH was adjusted by the addition of 2N HCl or 2N NaOH and its constancy was checked after each exit experiment by using a glass electrode, direct reading pH meter (EIL). No significant change in the half saturation (ϕ_I) was observed over the pH range studied.

The apparent ϕ_I was determined every 5°C over the range from 12°C to 37°C. Constant temperature was maintained in the cuvette by means of a surrounding water jacket through which water was circulated from a thermostatically controlled water bath. The temperature setting was adjusted at the beginning of each experiment and the temperature observed until it remained constant for a quarter of an hour. The temperature in the cuvette was checked before and after a reading was made: variations were rarely found to be more than 0.5°C, but if they did exceed this value the

Effect of Temperature on Phenolphthalein Inhibition of Glucose Exit in Human Erythrocytes.

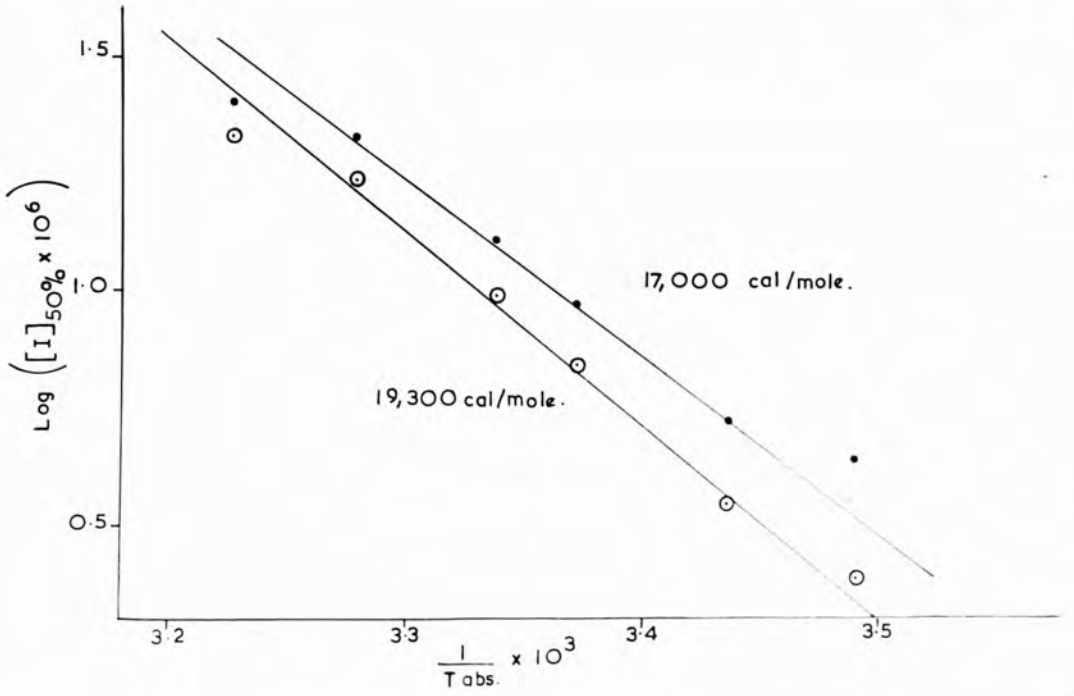


Fig. 4.2. - Upper line Arrhenius plot of the half saturation dissociation constants for phenolphthalein. The log of the half saturation constant is linearly related to the reciprocal of the absolute temperature, and the slope corresponds to 17,000 cal/mole for the extra energy required for the dissociation of the complex which phenolphthalein is postulated to form with the membrane component.

Lower line represents equivalent plot with a correction (see Appendix) made for competition with glucose.

Note: Suggestion of a sigmoid characteristic in this particular experiment was not a general observation.

experiment was rejected. A difficulty at the lower temperatures was the condensation of moisture on the outside of the cuvette, which seriously affected the light transmission. At the lower temperatures, therefore, two jets of air from a compressed air cylinder were played over the faces of the cuvette to keep it dry.

The Arrhenius plot of the results obtained in these experiments is shown in fig 4.2. The points are not truly co-linear and there may be more than one rate term involved. However, if the half saturation constant with the inhibitor is taken as a dissociation constant, then this slope (17,000 cal/mole) which is steeper than that for glucose, indicates a greater difference in the energies required for formation and dissociation of complexes.

If the results are corrected for the competition exerted by the glucose in the outside solution the line for the half saturation inhibition concentration would be as shown below and have a slightly greater slope of 19,000 cal/mole. The correction necessary is discussed in an appendix.

Phloretin - While phlorrhizin is effective in inhibiting glucose absorption in the kidney and intestine it is the aglucone phloretin which is the more active in the red cell. Its action has been studied by Wilbrandt (1950), Lefevre (1959) and Sen and Widdas (1962). Lefevre used it to evaluate the dissociation constants for various sugars using the transport mechanism and to

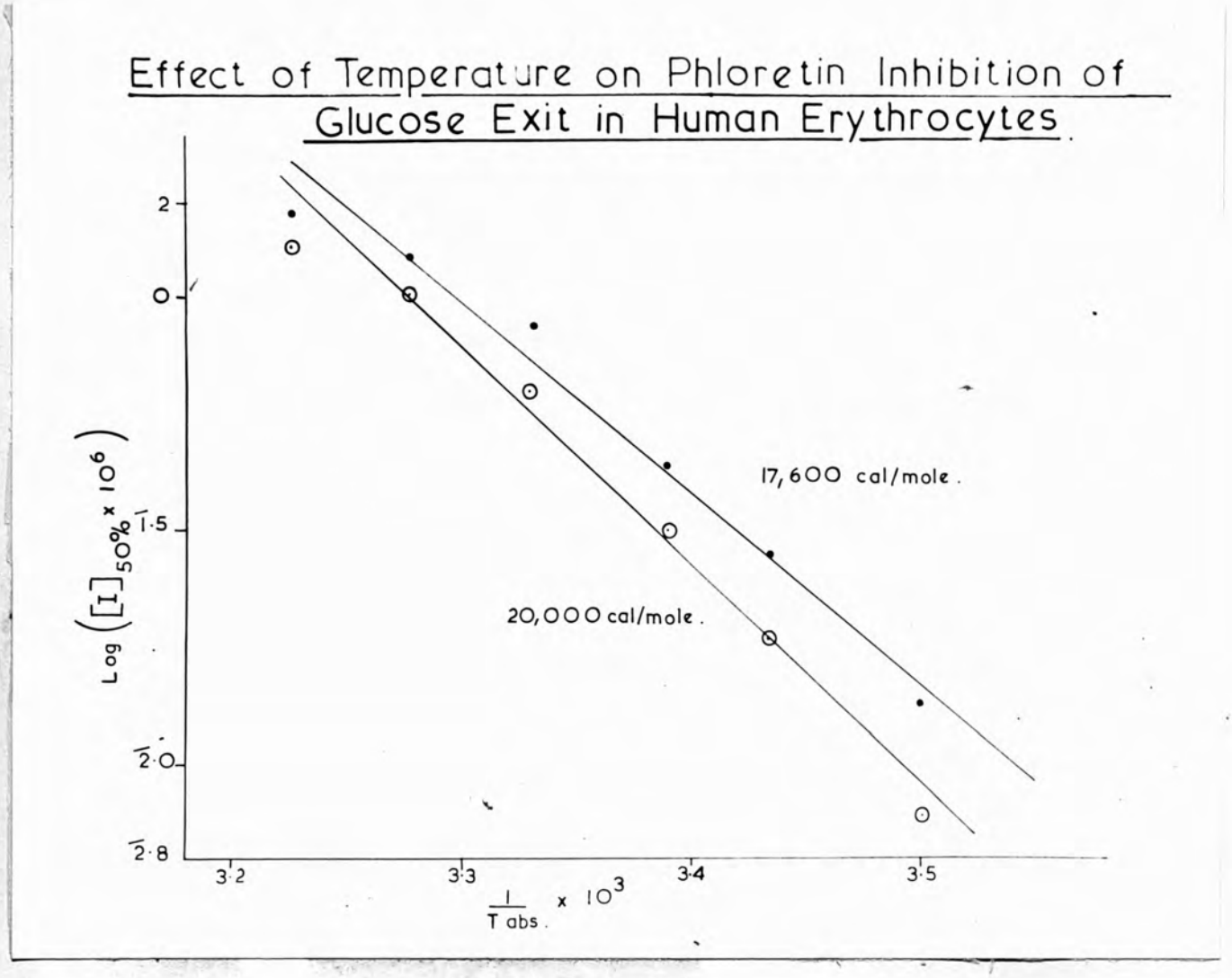


Fig. 4.3 Arrhenius plot of half saturation constant for phloretin.

The slope is 17,600 cal/mole.

Using the simple correction which allows for competition by glucose it becomes 20,00 cal/mole.

(c.f. Fig. 4.2.)

establish an order for them. He also examined the effect of pH on inhibitor uptake and inhibitory potency, finding that both fell as the pH increased. He ascribed this effect to keto-enol tautomerism, suggesting that only the ketonic form is directly reactive with the cell. The present experiments were carried out at pH 7.4 which lies in the middle of the range examined by Lefevre.

The variation with temperature of the half-saturation inhibitor concentration was obtained for phloretin as for phenolphthalein by following exits into varying inhibitor concentrations. The Arrhenius plot obtained is again not truly linear. The value of the slope 17,600 cal/mole is perhaps a little greater then for phenolphthalein, but sufficiently close to suggest that a similar mechanism is involved. Phloretin is, however, a considerably more potent inhibitor of glucose transfer. In agreement with Sen and Widdas (1962b) the concentration for 50% inhibition at 37°C was found to be 1.5×10^{-6} M whereas that for phenolphthalein was found to be 20 to 25×10^{-6}M. Lefevre found that any constraint between the two six-carbon rings reduces the potency of the diphenolic drug. The phthalic anhydride in phenolphthalein probably confers some restriction on the two rings and this may account for the lower potency of phenolphthalein when compared with phloretin

Effect of Phospholipids on inhibition by Phloretin - If a phospholipid were involved in glucose transfer, phloretin might be reasonably expected to enter into combination with such a

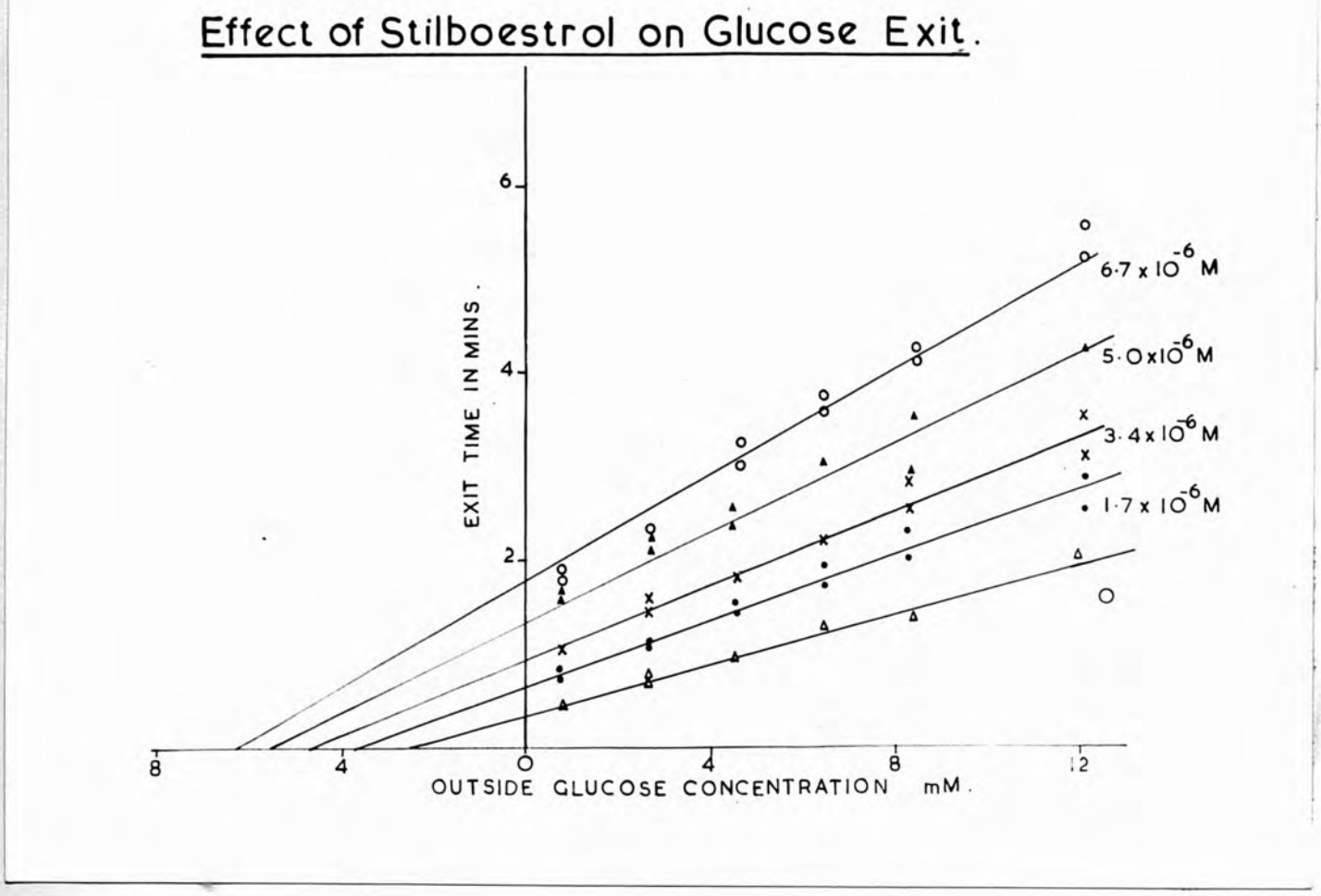


Fig. 4.4. - Plots of glucose 'exit' times for loss from cells, equilibrated with 76 mM glucose, into buffered saline media containing variation glucose concentrations in the presence of five stilboestrol concentrations in the range 0–6.7 x 10^{6}M. Two experiments were carried out at each inhibitor concentration.

(Temp. 37°C, pH 7.4).

substance. Exit of glucose into a phloretin-containing medium in the presence of suspensions of various phospholipids was, therefore, followed. Phosphatidyl inositol, cephalin, lecithin and cholesterol were used. Two mg of each lipid were dissolved in 1 ml. absolute alcohol and made up to 25 ml. with buffer. The cells were injected into the cuvette, containing 2 ml to 4 ml. of the lipid suspension and 2 ml. phloretin in a final volume of 21 ml. after previous equilibration in smaller volumes of similar solvents. The experiments were carried out at 33°C. Phosphatidyl inositol and phosphatidyl ethanolamine both gave a small reduction (about 10%) in the inhibition. Cholesterol gave a more pronounced reduction of inhibition (40 to 50%). The effect of lecithin could not be followed as it haemolysed the red cells. The action of the lipids in reducing inhibition by phloretin thus appears to have been non-specific. It probably represents the uptake of phloretin into the lipid micelles. This would be very likely to occur as phloretin is virtually insoluble in water.

Stilboestrol - Lefevre (1959) found stilboestrol to be the most potent of diphenolic inhibitors. In this drug full extension between the two phenolic groups is possible. Exit into varying concentrations in the presence of constant inhibitor concentrations was followed. The progressive increase in half saturation constant, as well known in enzyme studies and characteristic of competitive inhibitors, was seen. (fig. 4.4.)

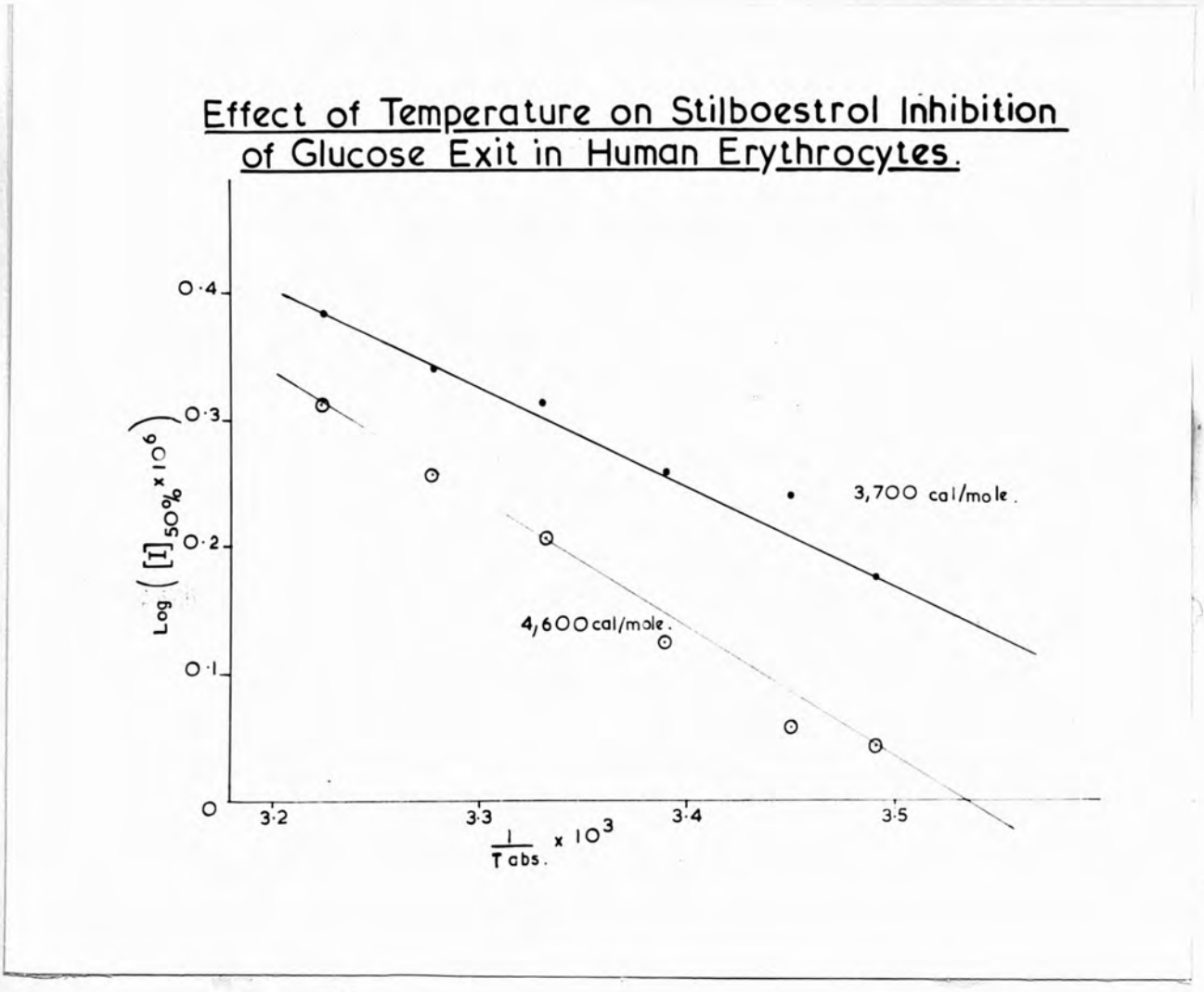


Fig. 4.5. - Arrhenius plot of half saturation constant for stilboestrol.

The slope is 3,700 cal/mole.

When the correction for competition is made this becomes 4,600 cal/mole.

Although stilboestrol is a competitive inhibitor the slopes are significantly less than for phloretin and phenolphthalein. (cf. figs. 4.2. and 4.3.).

Changing the pH of the medium over the range 6.5 to 8.4 was found to have little or no effect on the inhibition produced by stilboestrol.

The apparent half saturation constant was obtained at various temperatures and log ϕ_I plotted against the reciprocal of absolute temperature. A line was obtained with a slope of 3,700 cal/mole. (fig.4.5.) If the values are adjusted to take into account the presence of glucose, a slope of 4,800 cal/mole is obtained. In connection with these results it is of interest to note that the energy required to break two hydrogen bonds would be 6,000 cal/mole. The figure obtained is much lower than those for phloretin and phenolphthalein and could indicate a very much smaller difference in the energies for formation and dissociation of the complex. Alternatively there may be some other lipid solubility or conformational change with temperature which tend to keep the half-saturation more nearly constant.

Thyroxine - The effect of thyroxine on glucose absorption in the intestine has been studied by several workers. Althausen and his co-workers (1937, 1949) found that the thyroid stimulated glucose absorption. Moseley and Chornock (1947) could not find such an effect, while Ponz (1945) reported that thyroxine decreased sugar absorption. Levine and Smyth (1963) found that hyperthyroidism results in a greater uptake of glucose which does not, however, seem to result from alterations in hexose transfer. Since the molecule of thyroxine is in several respects similar to phloretin

the effect of thyroxine on glucose transfer in erythrocytes was studied, although inhibition might be contrary to its expected mode of action. Thyroxine was found to have no effect on glucose exit except to increase the extent of the excursion. It is probable that this molecule was ineffective because only one of the benzene rings was phenolic in character, the other being phenylalanine. Wilbrandt (1957) found that methylation of any of the four hydroxyl groups in phloretin resulted in a very considerable fall in inhibitory potency.

Results of Kinetic Studies.

CHAPTER 5.

Studies with non-competitive inhibitors of glucose transfer.

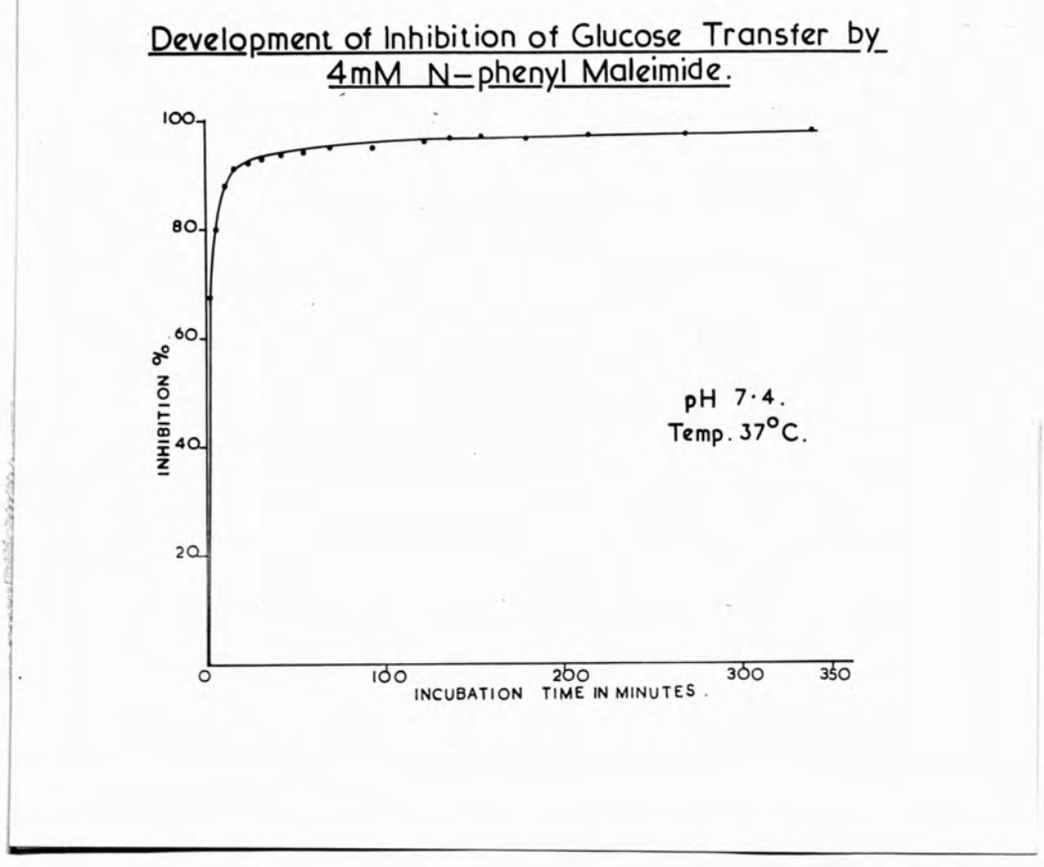


Fig. 5.1. Time course of development of inhibition by 4mM N - phenylmaleimide at 37°C and pH 7.4

The percentage inhibition of the maximal transfer rate $\frac{K_o - K}{K_o} \times 100$ has been plotted against time of incubation.

Effect of n-phenyl meleimide on glucose transfer.

It appeared from experiments performed on stilboestrol, to be described later, that the efficiency of an inhibitor of glucose transport across the erythrocyte membrane depenends to some extent upon its solubility in that membrane. N-ethyl maleimide (NEM) is quite a potent inhibitor of glucose transfer. Its homologue n-phenyl maleimide (NPM) containing a phenyl group would be relatively more soluble in lipid solvents than NEM and would possibly be expected to be more strongly absorbed and show greater potency as an inhibitor of glucose transfer. The action of 4mM n-phenyl maleimide was tested at 27°C, 33°C and 37°C. A solution was prepared of 14.3g NPM in 1 ml. absolute alcohol and made up to 10 ml. with buffered saline. For inhibition 3 ml. of this solution was added to 0.1ml cells in 2.9 ml. glucose-containing supernatant, after uninhibited glucose exits had been tested in the cells. Samples of 0.2 ml. were taken at various known times and glucose exit followed.

A record of the development of inhibition in one experiment is given in fig 5.1. There is a rapid initial inhibition followed by a slower progressive inhibition until transport was 97% inhibited with a small residual permeability remaining. Even after 6 hours incubation with the inhibitor, total inhibition was not achieved. The picture is similar to that obtained with NEM when a residual permeability of 3-5% is obtained. Dawson and Widdas (1963)

showed that for NEM residual permeability has the usual properties of facilitated transfer and does not represent a pathway for simple diffusion. They also showed that the reaction does not correspond to a pseudo first order reaction as would be expected if the inhibitor was in excess.

The progression of inhibition by NPM was affected to quite a large extent by temperature. This compound is effective as an inhibitor, but seems to be in no way more specific for glucose transfer processes than the previously used reagent NEM.

Effect of 1,5-difluoro 2,4-dinitrobenzene on glucose transfer.

Whilst the action of 1-fluoro-2,4-dinitrobenzene (DNFB) has been extensiveley investigated that of 1,5-difluoro-2,4-dinitrobenzene (FDNFB) has not been examined to any great extent. Berg et al (1965) have reported that treating human erythrocytes with a 3.0 mM solution of DNFB or 2.0 mM solution of FDNFB causes the cells to become permeable to sodium and potassium ions. Cells treated with the monofluoro reagent eventually lyse as a result of this increased permeability. Cells treated with the difluoro reagent did not haemolyse even after extraction with lipid solvents and were found to be remarkably rigid. They suggested that the difluoro compound formed cross links in the membrane and increased its strength. They also followed the permeability of treated cells to small water soluble non-electrolytes. They found that the permeability

of these substances fell in cells treated with the monofluro reagent but were not able to follow the penetration in cells treated with the difluoro reagent. It would be of interest, therefore, to see if its action could be followed using the method already described and to compare it with the effect of DNFB. This study might also throw some light on the mechanism of reactions within the membrane.

The development of inhibition was followed at 33°C and 27°C. A 1.0 mM solution of FDNFB was made in 10% alcohol in saline Care was needed because of the corrosive nature of this material. Packed cells (0.08 ml.), were incubated in a mixture containing 0.8 ml of FDNFB solution, 0.2 ml. 30% glucose and 3.13 ml. saline. Samples were withdrawn at convenient intervals and glucose exits tested. Almost complete inhibition was obtained after incubating for 1 hr at 33°C. After 5 hours it was not possible to obtain a record of glucose exit or of glucose entry but it was still possible to haemolyse the cells. FDNFB was taken up very readily by the cells and in quite large amounts. If a slightly greater quantity of cells were present the course of inhibition did not proceed to its full extent but levelled out at about 80%.

If FDNFB were exerting its effect by exerting cross links in the membrane, the mobility of the carrier should be affected so that the value of K would be changed. Therefore exits into varying concentrations of glucose were measured so that K and ϕ_g could be determined. The cells were incubated with the inhibitor

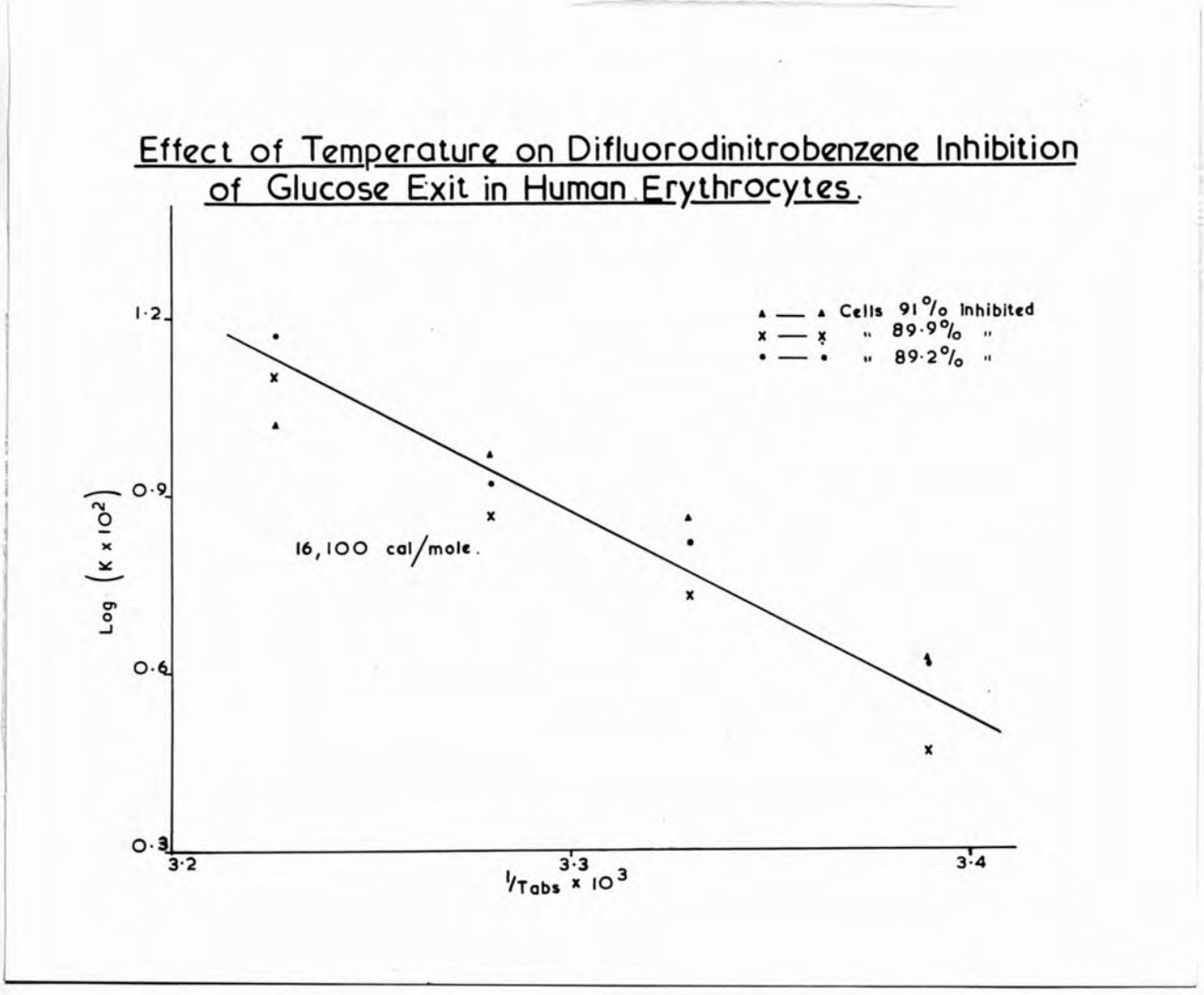


Fig. 5.2. - Effect of temperature on inhibition of glucose transfer in human erythrocytes by FDNFB. Log maximal transfer rate is plotted against reciprocal of absolute temperature. The results are from three experiments at approximately 90% inhibition. The scatter of points is probably due to the fact that the degree of inhibition was not exactly the same in the three experiments.
Slope of line is 16,000 cal/mole.

as described and when the required degree of inhibition had been reached (85-95%) the cells were spun down, washed twice with buffer containing 76 mM glucose and exits followed in the normal way. Experiments were carried out at 4 different temperatures, 22°C, 27°C, 32°C, and 37°C for three degrees of inhibition 85%, 90% and 95%. Values of K and ϕ_g were determined at each temperature for each of the three degrees of inhibiton and graphs of log K and log ϕ_g were plotted against the reciprocal of absolute temperature. ϕ_g was not affected by the presence of the inhibitor implying that the reaction between the sugar and the carrier was unaffected and suggesting that increase in the exit time was being caused by a reduction in the number of carriers available.

Plots of log K v. $1/T$ gave good straight lines; that for 90% is shown in fig. 5.2. Such scatter of points as is seen may be explained by the extreme difficulty of bringing the cells to the same degree of inhibition in different experiments. The slope obtained from the graph shown is 15,000 cal/mole. The values for 85% and 95% inhibition were 15,000 and 16,000 cal/mole. Sen (1960) found that in uninhibited cells a plot of log K against $1/T$ could be resolved into two straight lines: that over an upper range (32°C-47°C) had a slope of 6,800 cal/mole and that over the range below 32°C had a slope of 20,000 cal/mole. The values obtained here are of a similar order to those obtained by Sen for comparable temperatures so it seems that the inhibitor has not altered the steps in the passage of glucose across the membrane.

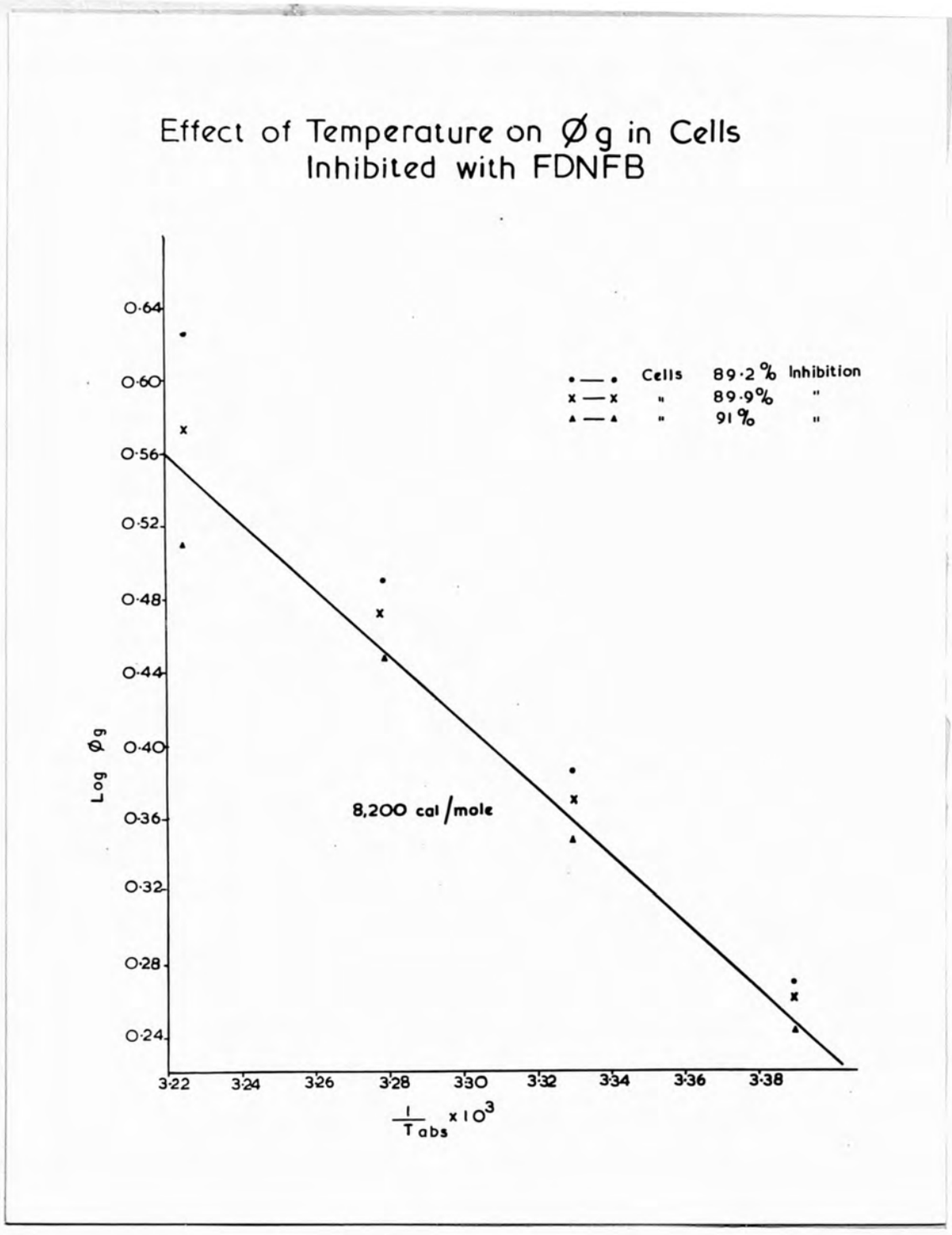


Fig. 5.3. - Plot of half saturation constant for glucose (Øg) in FDNFB treated cells against the reciprocal of absolute temperature.

Plots of log ϕ_g v. 1/T also gave quite good straight lines, that for 90% inhibition being shown in fig 5.3. For cells which were 85% inhibited the slope was 11,500 cal/mole. (Compare the value of 10,000 obtained by Sen and Widdas, 1962, for uninhibited cells). At 90% inhibition the figure is reduced to 8,200 cal/mole and at 95% to 6,200 cal/mole. Comparison within the present sequence of experiments, between the slopes for 85% and 95% inhibited fluxes suggests a significant trend. At 95% inhibition glucose would probably be entering chiefly through the small residual permeability found after inhibition. These results raise the possibility that the underlying mechanism in this case is different from that normally obtaining.

Exchange of Glucose in Inhibited Cells.

To check that the volume changes followed in these experiments were in fact a result of movement of glucose, the exit of ^{14}C-glucose was followed from cells inhibited with FDNFB. Concurrently the possibility of exchange diffusion was examined. Exchange diffusion describes a 1:1 exchange of substance across the membrane, as a result of free substrate molecules exchanging with those bound to the carrier. This is not to say that the process is limited to a situation where only complexed carriers move, but can be observed when the carrier complex is more mobile in the membrane as described by Britton (1964), Levine et al. (1965), and Mawe and Hempling (1965). One important feature in exchange diffusion is

that in contrast to simple diffusion, the flux of a substance in one direction is sensitive to the concentration of the substance in the compartment to which it moves. The flux of a sugar from loaded cells will occur at a lesser rate into a saline medium than into a sugar containing medium where loaded carriers may return to the inside face of the membrane.

Other models are possible involving two different molecular species of carrier, one capable of movement only when complexed but the other mobile in both complexed and uncomplexed states. Such models cannot be formally excluded but in the present state of knowledge they are not demanded. Heinz and Walsh had in 1959 found exchange diffusion of amino acids in Ehrlich tumour cells. Lacko and Burger (1961, 1962 and 1963) working at low temperatures were the first to indicate that the phenomenon was operative within the glucose transfer of the erythrocyte. It has subsequently been examined by several workers but it was felt that FDNFB inhibited cells would be a useful preparation for further study since the sugar movement was so much reduced as to allow easier time resolution. The possibility that the residual fluxes are of a different kind from the majority fluxes is not excluded.

To follow movements of the isotope the following procedure was adopted. Twelve millilitre packed cells were incubated with 76 mM glucose (final volume of solution 110 ml.) at 37°C for 30 minutes. A sample was taken for exit, the cells spun down and 10 ml. packed cells were incubated with 30 ml. saline, 60 ml

inhibitor solution (104mg/100ml.) and 10 ml. 30% glucose solution until the glucose exit was 90% inhibited. The cells were spun off and washed twice with ice-cold buffer containing 76 mM glucose. The percentage inhibition after washing was checked. Eight millilitre of the cells recovered were incubated at 37°C for one hour with 30 ml. 76 mM glucose solution containing only unlabelled glucose and 10 ml. 76 mM glucose with ^{14}C-glucose of total activity 10μC. From this mixture two 0.2 ml samples were taken to 50 ml. distilled water for counting and haemoglobin determination (see below). The cells were once more spun down and two samples of 0.2 ml. taken of the supernatant for radioactive counts. The difference between this count and the previous one provides a figure for cell content at zero time.

The cells were now taken up in a very small volume of supernatant and at varying intervals 0.2 ml added to one of a series of eight tubes containing either 4 ml. saline or 4 ml. 76 mM glucose. When the ^{14}C-glucose loaded cells had been in the fresh suspension for the required length of time, glucose movement was stopped with 10^{-3} M mercuric chloride and the cells yet again spun down. Results of control experiments in which the cells were spun down without prior inhibition were not, however, significantly different. From the supernatant 0.2 ml. samples were removed for counting as a check on glucose movement and cell content of glucose. Of the cells resuspended in a small volume of supernatant, 0.2 ml were

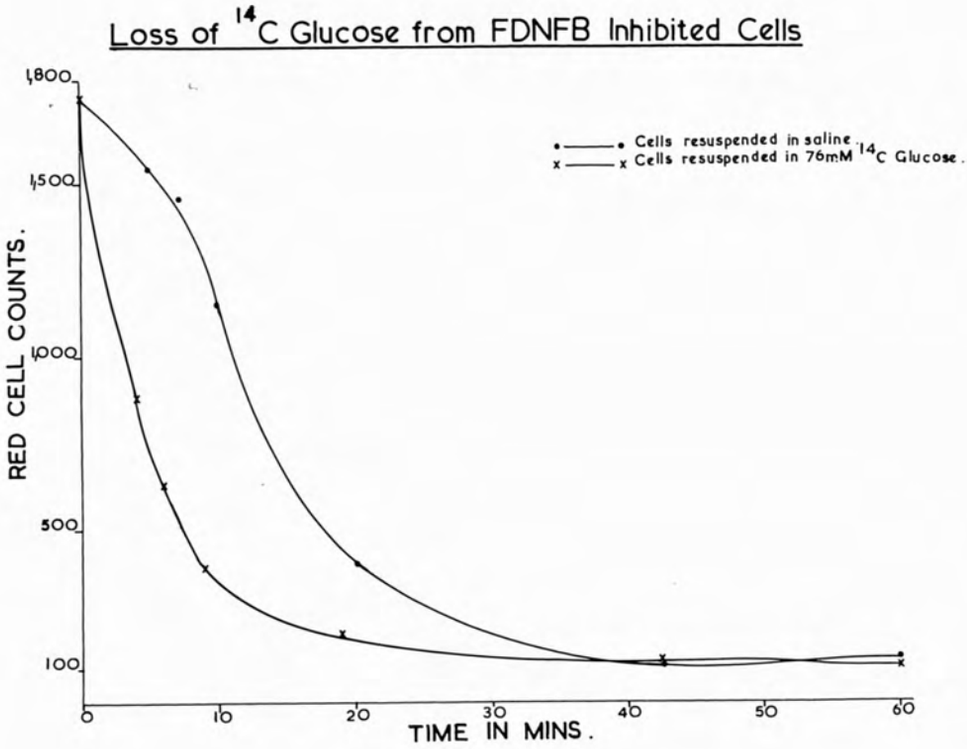


Fig. 5.4. - Plot showing efflux of glucose from FDNFB inhibited cells into saline and glucose saline.

Counts remaining in cells plotted against length of time in resuspension.

spun down alone so that the counts initially present in the cell could be checked against previous estimates. All samples were then haemolysed with 2 ml. distilled water and haemoglobin estimation effected by taking 0.05 ml. aliquots and diluting them a further 200 times for spectrophotometric estimation at 415 mμ. The proteins were precipitated as described by Harris (1964), 1 ml. 1% barium hydroxide and 1 ml. 1:1% zinc sulphate were added to the haemolysate in each tube, and the precipitate spun down. Two samples of 0.2 ml . were taken from the supernatant for counts. The amount of ^{14}C-glucose present in the cells after introduction to the sugar or saline solutions was then calculated.

^{14}C-glucose was able to penetrate FDNFB treated cells quite readily and its exit was quite easily followed by the method described above. As expected the movement of ^{14}C-glucose from the cell was considerably enhanced by the presence of glucose in the external medium. Fig.5.4. shows the exit of glucose from 90% inhibited cells into saline and glucose containing saline. In the presence of glucose the isotopically labelled sugar moves out very rapidly initially, the time for 50% of the sugar to leave being about three minutes and effective equilibrium being achieved after the order of 25 minutes. In the presence of glucose free saline 50% of the sugar had been lost from the cells after 12 minutes and effective equilibrium was reached after 40 minutes. Cells were examined at various degrees of inhibition and on average the exit

of 50% of the glucose was six times faster into a glucose containing solution than into saline alone.

Glucose Sorbose Exchange - As mentioned in the introduction, the values of $\emptyset g$ obtained, following sorbose entry in the presence of glucose were twice those obtained for exit experiments. This could be explained if glucose bound to the carrier exchanged with sorbose. A series of observations was, therefore, made with 76 mM sorbose in the external solution. The results (not shown) were surprising in that the exit was slower than for glucose, if anything in the few experiments performed, even slower than into saline. As the affinity of sorbose for the carrier is low, the result is contrary to immediate expectations but it could be explained if the reaction between the carrier and sugar were enzymically controlled.

To achieve a constant degree of inhibition and possibly more comparable results, for exchange experiments NPM could be used and the residual permeability, about 3%, used in such studies.

Results of Kinetic Studies.

CHAPTER 6.

Studies with non-glucose substrates and modified saline media.

Studies with Non-glucose Substrates.

Experiments with Galactose.

Of the several sugars which appear to enter the human erythrocyte using the same carrier as glucose, galactose has been widely studied. It is an isomer of glucose, differing only in the relative positions of the hydrogen and hydroxy group on carbon atom four.

LeFevre and Davis (1951) and Wilbrandt (1957) reported that it has a lower affinity for the carrier than glucose. Its entrance into muscle cells has been observed by Levine and into the cells of the mucosa from the intestinal lumen by Fisher and Parsons (1953). Newey, Sandford and Smyth (1965) have suggested that is shares one of the two available routes for glucose absorption in the intestine. It was considered that it would be of interest to study the entry of this sugar into the red cell further - to determine the effect of temperature and also phenolphthalein upon its half saturation constant.

Galactose exits were followed into a solution containing increasing concentrations of galactose over a range of temperature 12°C to 37°C. The affinity of galactose for the carrier was found to be less than that of glucose. The half saturation constant at 37°C was 14.2 mM, some four and a half times greater than that for glucose. A plot of the log of the half saturation constant against the reciprocal of absolute temperature gives a slope in the region of 7,700 cals/mole indicating a lesser difference

between the energies of association and dissociation than for glucose which would be expected as association does not appear to occur with the same facility as for glucose.

Exit of galactose was followed into solutions containing increasing amounts of phenolphthalein. Galactose exit appeared to be inhibited in the same way as that of glucose had been. The concentration of phenolphthalein required to double exit time at $32^{\circ}C$ was 21×10^{-6} M, the same concentration as found for glucose.

Experiments with 2-Deoxyribose.

It is probable that all sugars do not enter the erythrocyte by the same mechanisms. Steinbrecht and Hoffman (1964) have found that in the rabbit erythrocyte 2-deoxyribose enters by a process exhibiting diffusion kinetics, but with a half saturation constant of 1M. The rate of uptake appeared to be unaffected by glucose, ribose, or xylose, DNFB, phloretin, $CuCl_2$, $HgCl_2$ and iodoacetate, all at various concentrations. As this was a novel observation experiments were performed to examine the passage of this substance across the human erythrocyte membrane.

A 30% solution of deoxyribose consisting of 1 gm in 4.5 ml. buffer was prepared and entries followed after an injection of 0.5 ml. of this solution into the cuvette. A fuller account of techniques for entry experiments is given in Chapter 8 pages 123-124 It was found that a more satisfactory record could be obtained if 0.25 or 0.5 ml. of 30% glucose was first injected into the cuvette.

The 2-deoxyribose entered slowly and appeared to do so by diffusion. Exits were quite easily followed, although the linear portion of the record was slowed as compared with that for glucose. It was found that increasing the glucose in the cuvette over the range 2.7 to 12.2 mM had only a slight effect, the exit time showing a very small increase. Incubation with ~~with~~ 8 mM NEM appeared to have no effect on the exit time. Thus the observation of Steinbrecht and Hoffman was to some extent confirmed in the human erythrocyte. 2-deoxyribose has only four carbon atoms in the ring so that it is very possible that it could enter by diffusion.

Experiments using Modified Saline Media.

The evidence for sodium dependent glucose transfer in the small intestine is well documented (Crane 1960; Smyth 1963, Quastel 1965). As glucose transfer has also been found to be dependent on the presence of sodium in other tissues - for example the diaphragm (Clausen 1965), it was felt that it would be of interest to see if reduction or absence of sodium in any way affected glucose transport in the erythrocyte. A few experiments were carried out to eliminate sodium as far as possible.

Attempts were made to replace the sodium by inositol. A 0.3 M solution was prepared containing 5.4g/100ml. Exits were performed in the normal way into a medium containing increasing quantities of inositol. Exits could satisfactorily be obtained until 45% inositol was present. No satisfactory record could

then be obtained. The cells were not haemolysed and appeared normal under a phase contrast microscope. The instability of the cells was probably due to the large potentials which could arise when the ionic distribution was disturbed. It was found that the half saturation constant for glucose was quite normal when 25% of inositol was present in the cuvette.

Sodium was replaced entirely in the buffered solution by potassium. Stock solutions were prepared of KCL 12.7g/100 ml. and KH_2PO_4 3.51g/100 ml. These were diluted and used in the same way as the equilivalent sodium containing solutions. Exits were followed in the usual manner. The half saturation constant did not appear to be affected by the absence of Na^+. There was thus no evidence that a dependence similar to that in the intestine existed.

Results of Uptake and Extraction Studies using ^{14}C-labelling.

CHAPTER 7.

Uptake of ^{14}C-labelled inhibitors by human erythrocytes.

Uptake of Inhibitors by Erythrocytes.

As mentioned in the introduction, inhibition of glucose transfer by NEM and DNFB exhibits several interesting properties. Even though, as Sen and Widdas (1962b) reported, the half saturation constant for glucose remains unchanged in cells progressively inhibited by DNFB, the most likely explanation of its action is that it complexes in some way with the glucose transfer system. NEM is essentially similar, thus it appeared likely that these irreversible inhibitors would provide useful labelling of the proposed carrier system. Uptake of ^{14}C-labelled inhibitors by the cell was followed therefore and correlated with the inhibition produced. It was hoped that this would give some information as to the nature of the reaction involved and indicate the concentration of components which could be expected in proposed isolation experiments.

Essentially the procedure in these experiments was to incubate the cells with the inhibitor and as inhibition progressed to remove samples for simultaneous determination of percentage inhibition and quantity of inhibitor bound to the erythrocyte.

Binding of NEM and Inhibition of Sugar Transport.

Preliminary experiments in which the development of inhibition by NEM in varying concentrations at 21°C was followed indicated that 6.0 mM and 4.0 mM would be suitable concentrations for the proposed experiments.

From the solution of radioactive material supplied, 1.25 mg.

in 1 ml. alcohol, 0.2 ml. was added to 2.8 ml. buffer for spectrophtometric determination. It had been found that NEM could satisfactorily be estimated in the range 0.05 - 1.0 mM by reading the absorption at 300 mμ. Two stock solutions of 5 ml. were prepared.

a.) - 0.5 ml. ^{14}C-NEM + 9.375 mg unlabelled NEM
0.25 ml. 30% glucose

b.) 0.2 ml. ^{14}C-NEM + 4.75 mg unlabelled NEM
0.25 ml. 30% glucose solution.

From each of the stock solutions 1 ml. was taken for the concentration to be estimated and radioactive counts determined. The remainder was used for incubation with the red cells.

The erythrocytes were equilibrated with 76 mM glucose as for normal exit procedure and the exit time in uninhibited cells determined.

Incubation mixtures were then set up with :

4 ml. NEM solution

3,84 ml. sugar containing supernatant.

0.16 ml. packed red cells.

Two samples of this cell suspension were removed at four varying intervals from the commencement of incubation over a period of 210 minutes.

One sample (a) of 0.4 ml. was used for duplicate determinations of the exit time and the other (b) of 1.0 ml was taken to follow

the binding of radioactivity in the following way:- The sample was added to 9 ml. of ice cold buffer, mixed well, divided into two small pyrex tubes and spun down; 0.2 ml. of supernatant was taken for radioactive counting and the remaining supernatant discarded. The cells were resuspended in 5 ml. buffer and spun down again and planchets made of the supernatant. The last procedure was repeated so that the cells were washed three times. The cells were resuspended in 1 ml. saline of which two samples of 0.2 ml. were taken onto planchets and two of 0.2 ml. for haemoglobin estimation as described in Chapter 5.

To determine that the NEM complex was stable and the process leading to its formation truly irreversible, a further sample of 2 ml. was removed at five minutes in the second experiment where 4.2 mM NEM was used. The cells were washed twice, resuspended in a 76 mM glucose and incubated at 21°C. The time for exit was tested at 60 and 120 minutes, and at 121 minutes a 1 ml. sample was taken and treated as for "b" above.

The degree of inhibition produced was determined from the exit times as described by Dawson and Widdas (1963). By comparison with the counts of the standards, the uptake of NEM could be expressed in micro-moles NEM and knowing the haemoglobin concentration this could be expressed in micro-moles per cell.

Fig. 7.1. shows the development of inhibition of glucose transfer and the uptake of NEM at the two concentrations. The dotted lines represent results obtained for sample X, i.e. those

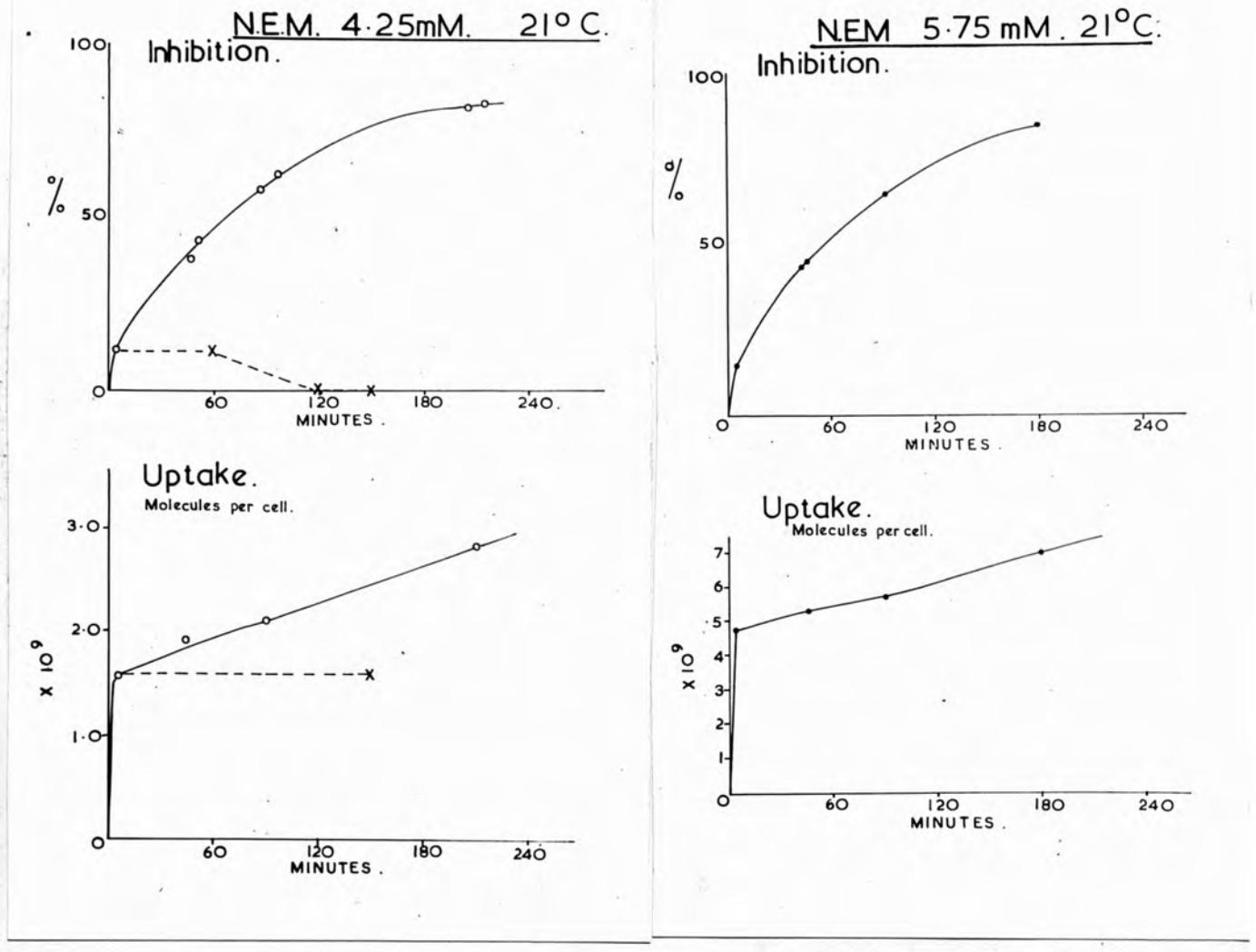


Fig. 7.1.- Inhibition of glucose transfer in human erythrocytes at 21°C / NEM.

Concentrations used were:-

1) 4.25 mM 2) 5.75 mM

Above - percentage inhibition plotted against time of incubation.

Below - uptake of NEM in molecules per red cell.

Broken line represents sample removed after 5 minutes and subsequently incubated in NEM free medium.

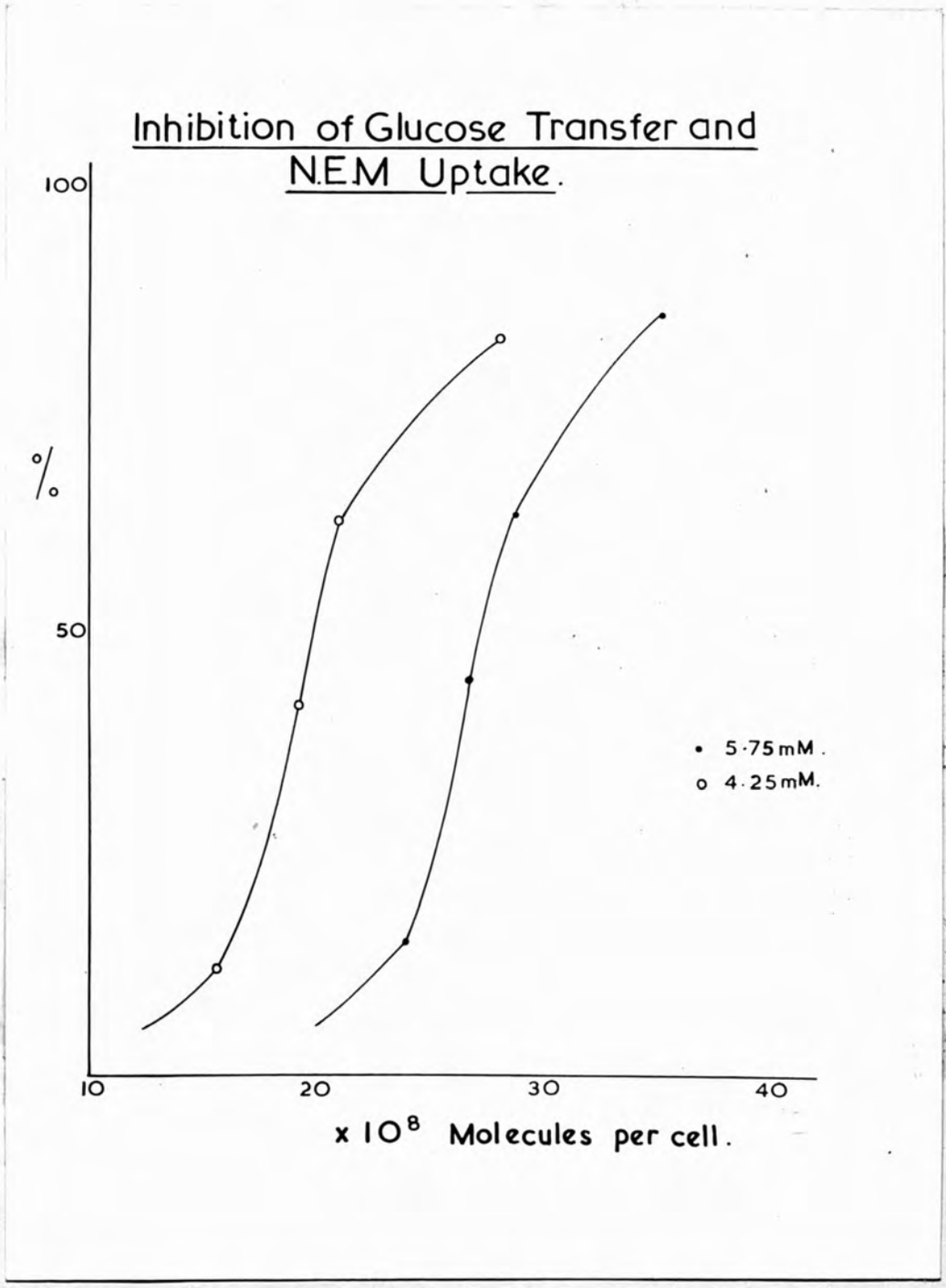


Fig. 7.2. - Inhibition of glucose transfer in red cells by 4.25 and 5.75 NEM at 21°C. Percentage inhibition is plotted against uptake of NEM in molecules per cell.

red cells removed after five minutes and incubated in NEM free medium. The labelling in this sample remains constant, although inhibition appears to fall off after 120 minutes. However, the times for exit at this low degree of inhibition were small and the difference may be due to error in measurement. It may be seen that there is a very rapid initial uptake of labelling whilst inhibition develops at a slower rate. After five minutes the uptake drops to a much slower but uniform rate, whilst inhibition develops at a fairly constant rate.

There is a sigmoid relationship between uptake and inhibition, (fig. 7.2.). The steepest part of the curve gives a slope which suggests that 1×10^9 molecules NEM per cell would be required to produce full inhibition of glucose transfer. This would represent labelling of quite a large component in the cell and must be considered an overestimate.

Binding of DNFB and Inhibition of Sugar Uptake.

The procedure was the same as in the experiments using NEM except that constant inhibitor concentration was used and the temperature of incubation varied. These experiments were carried out in conjunction with Professor W.F. Widdas and Miss J.C. Remfry, and have been briefly reported (Forsling, Remfry and Widdas, 1964).

Bowyer and Widdas found that low concentrations of DNFB of the order of 1 mM gave almost complete inhibition after several hours incubation with red cells with little risk of haemolysis. After several experiments it was decided that 1.4 mM DNFB gave a suitable development of inhibition.

^{14}C-DNFB, 2.1 mg, (specific activity 23.5 µc/mg) was added to 98 mg unlabelled DNFB and made up to 19.8 ml. with absolute ethyl alcohol

For the experiment 0.66 ml. of this stock solution were diluted to 6.3 ml. with a 76 mM solution of glucose in buffered saline, of which 1 ml. was made up to 10 ml. to be used as a standard.

Incubation mixtures were set up:-

5 ml. DNFB solution

4.8 ml sugar containing supernatant

0.2 ml. packed red cells.

The experiments were performed at 15°C, 21°C, 28°C, and 33°C In the first experiment at 21°C the yields of radioactivity were lower than expected so that a loss of DNFB by evaporation was suspected. To prevent this cysteine (a solution containing 30 mg/25 ml.) was used to bind the free DNFB. The DNFB containing supernatant was mixed with the cysteine solution in the proportion 5:1 or 0.2 ml. samples were pipetted onto the planchets already containing 0.1 ml. cysteine. Since little radioactivity was lost in the washing, samples were not taken of the final washing.

In fig. 7.3. results from experiments at 15°C and 33°C are plotted. As before the dotted lines show results from sample X, which here shows no change in the degree of inhibition nor in the amount of radioactive labelling bound, confirming the irreversibility of the combination. Both the uptake and inhibition proceeded at a slower rate at the lower temperature, but similar uptake results are obtained at both temperatures so that uptake appears independent

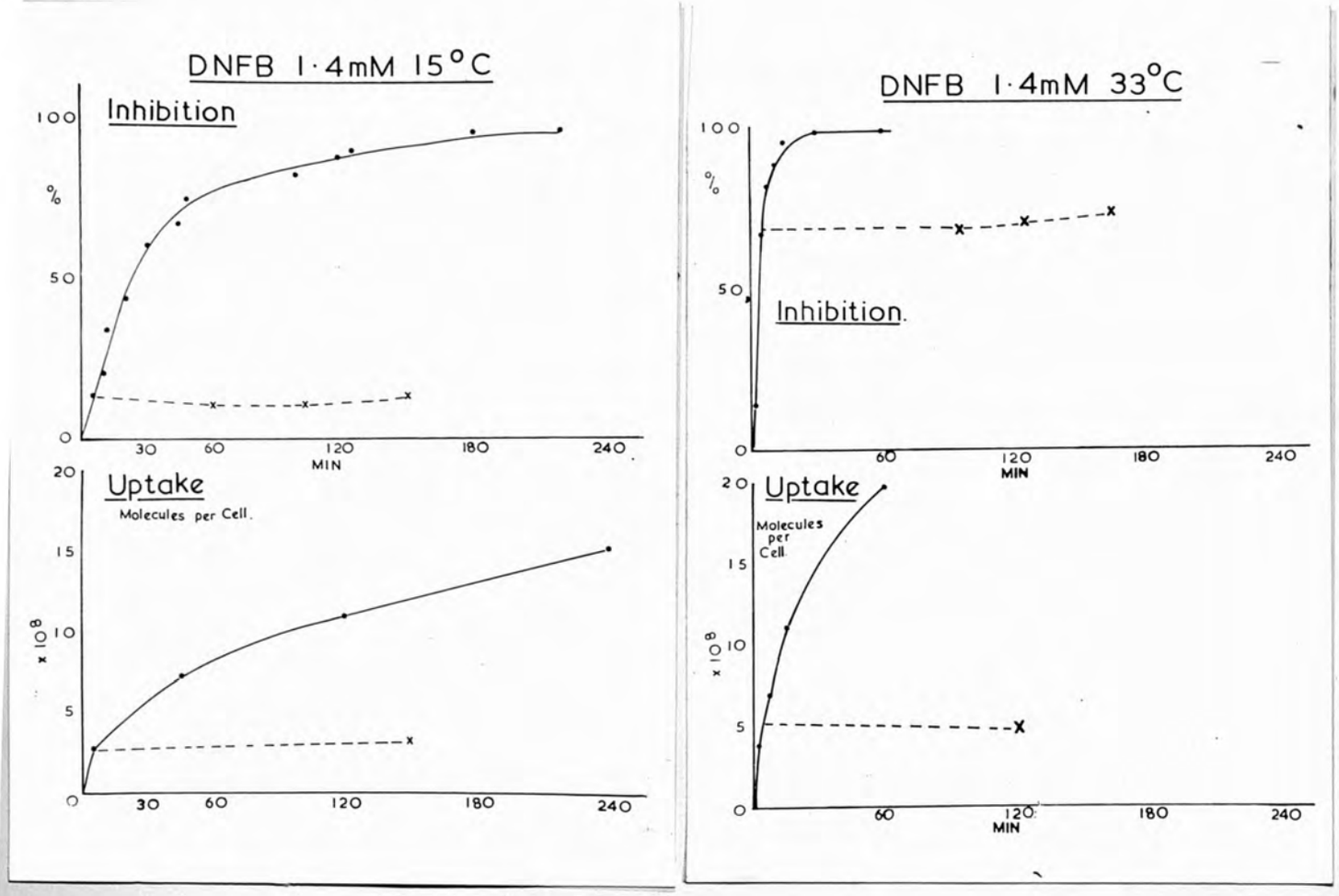


Fig 7.3. Inhibition of glucose transfer in human red cells by 1.4 mM DNFB at

(1) 15°C (2) 33°C

Above - percentage inhibition plotted against time of incubation.

Below - uptake of DNFB in molecules per red cell.

Broken line represents sample taken after 5 minutes and subsequently incubated in DNFB free medium.

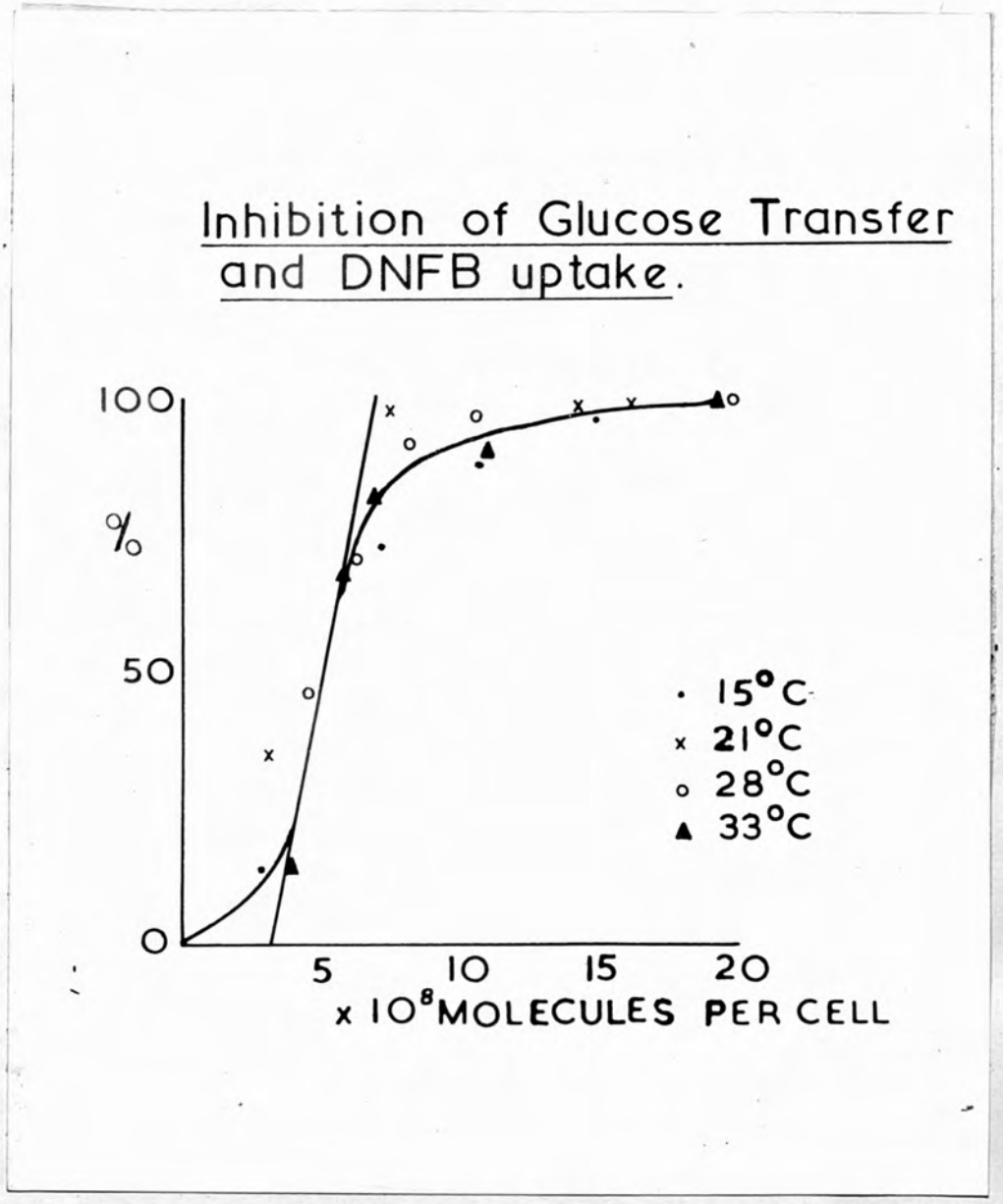


Fig. 7.4. - Inhibition of glucose transfer in red cells by 1.4 mM DNFB.

Percentage inhibition is plotted against uptake of DNFB in molecules per cell.

Incubations at 15, 21, 28 and 33°C.

of the rate of development of inhibition as may be seen from fig. 7.4., which summarises the results obtained. The relation between inhibition and uptake is also seen to be independent of temperature.

From the steepest part of the curve it seems that an uptake of 4×10^8 molecules DNFB per red cell would be required to produce full inhibition of glucose transfer. This is some two and a half times less than the value obtained for NEM but a comparison between the two experiments is not truly valid as the nature of the processess involved is not really understood, and for each compound there may be a different mechanism as the basis of action. No "fixer" was used in the NEM experiments so that while the radioactivity bound to the cell was unaffected, that in the standard solution might have been affected by evaporation so giving a falsely high value for radioactive uptake by the cell. It was also later reported that the ^{14}C-NEM used in subsequent experiments had deteriorated. If this were the case in these experiments, part of the radioactive uptake would represent a non-specific uptake of breakdown products of NEM. Both the values for NEM and DNFB must obviously be regarded as an overestimate since the inhibitor does not bind only with the membrane carrier sites during the development of inhibition. It has been estimated that the -SH groups of haemoglobin comprise about 85% of the total reactive thiols of the normal human erythrocyte, glutathione 10% and the membrane less than 5% Morell, et al. (1964), studying the reaction of NEM with intact erythrocytes obtained similar values.

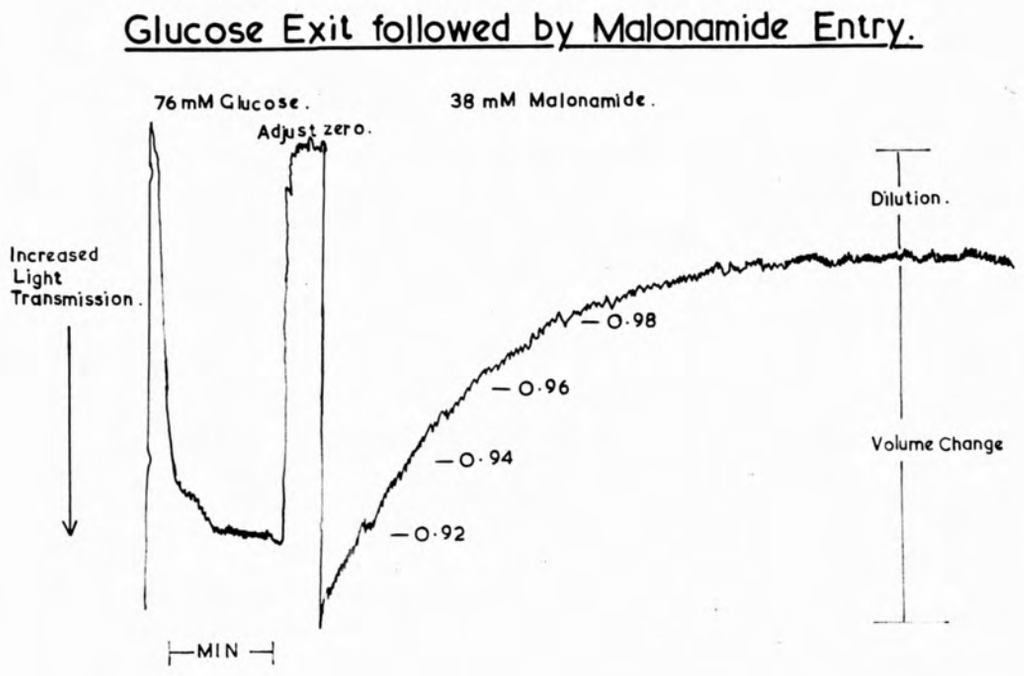


Fig.7.5. Tracing of a recording of glucose exit from cells equilibrated with 76 mM glucose followed by entry in 38 mM malonamide. (37°C pH 7.4)

Weed, et al., (1962, 1964), studied the interaction of mercury and mercurial compounds in conjunction with studies of inhibition of glucose transfer loss of potassium and osmotic fragility. They give a slightly different distribution of thiols, 90% in haemoglobin, 6% in glutathione and 4% in the membrane. Glucose transfer was inhibited when 1.2% of the membrane SH groups had reacted and from this they calculated that the maximum number of transport sites in the intact cell is 700,000 per cell and that the number of glucose molecules would cover about 1% of the red cell surface.

Effect of DNFB on Malonamide Entry into Erythrocytes.

From these results it seemed that ^{14}C-labelled DNFB and NEM would be of value in attempts to isolate the components with which glucose is presumed to form a complex. It is, however, possible that DNFB has a non-specific effect on the membrane simply forming cross links and "stiffening" the membrane, like tannic acid (Hunter, 1964). To determine if this possibility could be eliminated the effect of DNFB on the penetration of malonamide into the cell was studied. Malonamide enters the cell slowly and appears to do so by diffusion so that its entry would be affected by drugs acting generally on the membrane

The entry was followed in the photoelectric apparatus. Cells were equilibrated with 76 mM glucose and glucose exit from these cells into glucose-free medium was followed at 37°C. The pen was then adjusted to record at the top of the paper and 1 ml. of a 15% solution of malonamide was then injected into the cuvette. The light transmission rapidly increased owing to the dilution of

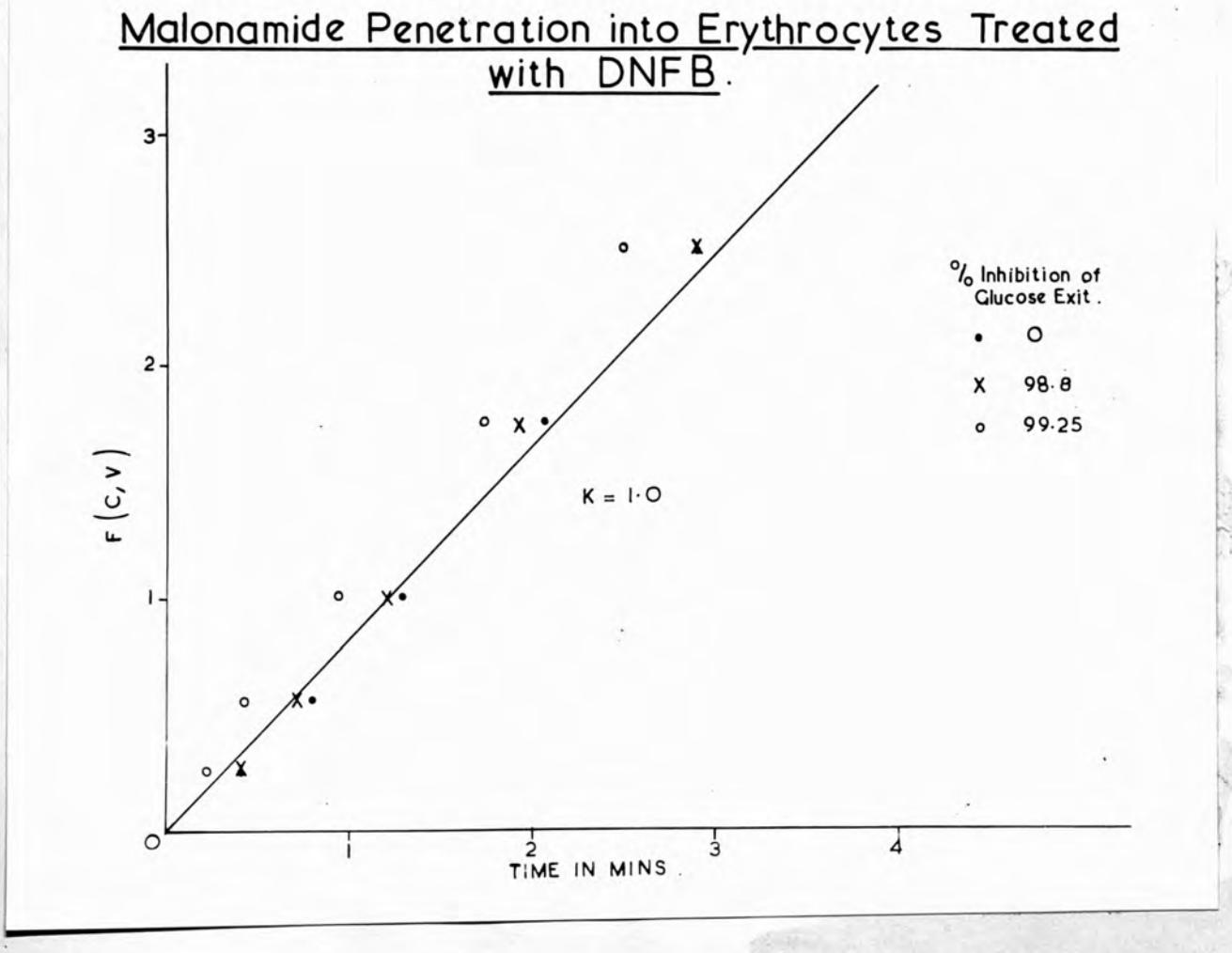


Fig.7.6. Malonamide penetration into erythrocytes in which glucose exit was inhibited 98.8% and 99.25% by 1.4 mM DNFB. Times to reach specified volumes have been plotted against appropriate values of the function F (CV) (Widdas 1954) so that the slope of the line drawn by eye gives the value of K the penetration constant on the basis of diffusion.

the cell suspension and the shrinkage of the cells in the hypertonic mixture of saline and malonamide. As the malonamide entered, the cells began to swell and the light transmission increased as may be seen in the record (fig 7.5.).

The cells were then incubated in a 1.4 mM DNFB solution for a time sufficient to produce 98-99% inhibition, when glucose exits followed by malonamide entries were observed.

The results were calculated as described by Widdas (1954). The intermediate volumes 0.92 Vi, 0.94 Vi, and 0.98 Vi were marked off on the record and the time taken for the suspension to return to these volumes was measured. These times were then plotted against functions calculated on the basis of diffusion (fig 7.6.) from which it may be seen that the rate of penetration of malonamide was unaffected by DNFB.

Uptake of ^{14}C-Stilboestrol.

LeFevre (1959) found stilboestrol to be a potent inhibitor of sugar transfer in the human erythrocyte. Its apparent high specificity would suggest that it could be used to label the membrane component involved in transfer. Experiments described earlier indicate that this compound is, as supposed a competitive inhibitor with a low apparent half saturation constant. As the action is competitive the technique adopted for correlation of uptake and inhibition by NEM and DNFB could not be used and instead it was decided to attempt a determination of the reduction in uptake, if any,

Table 7.1.

Uptake of Stilboestrol by Red Cells in presence of increasing quantities of glucose. Distribution of Counts between Cells and Supernatant.

		Experiment 1		Experiment 2	
Series	Tube Number	Cells cts/ml.	Supernatant cts/ml.	Cells cts/ml.	Supernatant cts/ml.
1	1	223,000	672	1,052,000	1,285
	2	214,000	1,094	1,191,000	3,116
	3	257,000	1,320	1,212,000	3,731
	4	327,000	1,649	1,188,000	4,195
	5	237,000	1,946	913,000	5,565
Series 2	1	409,000	950	1,158,000	1,913
	2	333,000	1,122	1,167,000	2,997
	3	457,000	1,510	1,194,000	4,541
	4	463,000	1,902	1,075,000	5,132
	5	448,000	2,236	942,000	6,682

produced by saturation of the carrier to a lesser or greater extent with glucose. The studies were performed over a wide range of sugar concentrations which would allow the blocking of an estimated 18 to 60% of the available sites.

The radioactive stilboestrol, 1.786 mM in 0.1 ml. benzene was taken to dryness and dissolved in 4 ml. absolute ethyl alcohol.

A series of five tubes was set up with 0.3 ml. packed cells in phosphate buffer containing 0, 2.5, 10, 25, and 50 mM glucose. The tubes were incubated for three quarters of an hour at 37°C; 0.1 ml. (containing 4.47 n moles) of the alcoholic solution of stilboestrol were added to each tube which was thoroughly shaken. After ten minutes the solution was transferred to a 1.5 ml. haematocrit tube with a 0.1 ml portion of narrower bore for the cells and spun at 3,000 r.p.m. for five minutes. Part of the supernatant was removed to a small tube and two 0.05 ml. samples taken for counting. The remainder of the supernatant was removed leaving only a trace on top of the cells. To wash, more saline was added to the top of the haemotocrit tube and pipetted out, removing the top layer of cells also. Distilled water, 1.0 ml. was added to the haematocrit tube and 0.1 ml. of the laked cells taken for counts and 0.05 ml. for haemoglobin determination . A repeat experiment was carried out for thirty minutes after incubation commenced (series b). As can be seen from the results, the cells were not in equilibrium with the stilboestrol solution when the first set of readings were obtained and the counts in the second series were considerably

higher. A similar experiment was carried out using 0.1 ml. of cells and 0.2 ml. stilboestrol. The first sample was then taken after fifteen minutes and the second after forty minutes.

The results showed that there is a large uptake of stilboestrol which does not appear to be affected by the presence of glucose. The fall in counts in the supernatant seems not to be significant as some counts were probably absorbed by the thin film of sugar found on the planchets. Experiments showed that counts declined if more than 5 mg of sugar were present, which was the case in experiments at high glucose concentration. This could be overcome by isolating the stilboestrol in ether before counting. Since this large apparently non-specific uptake was observed, it was decided to study the effect of concentration of stilboestrol present in the incubation medium on uptake by cells.

A stilboestrol solution containing 50 µc in 4 ml. was made and a series of cell suspensions (1-5), of a total volume of 4.5 ml. containing 0.1 ml packed cells and 0.05, 0.2, 0.4, 0.6 and 0.8 ml. of stock stilboestrol solution, were prepared. Ten minutes after the addition of stilboestrol the cells were spun down in a haematocrit tube for five minutes at 3,000 r.p.m. The supernatant was removed as cleanly as possible and 2 x 0.2 ml. taken for counts from the first two tubes (containing relatively low concentrations of stilboestrol) and 0.05 ml. from the other tubes. To test for haemolysis, which would interfere with stilboestrol determination 0.75 ml. supernatant was taken up in 1.75 ml. of distilled water.

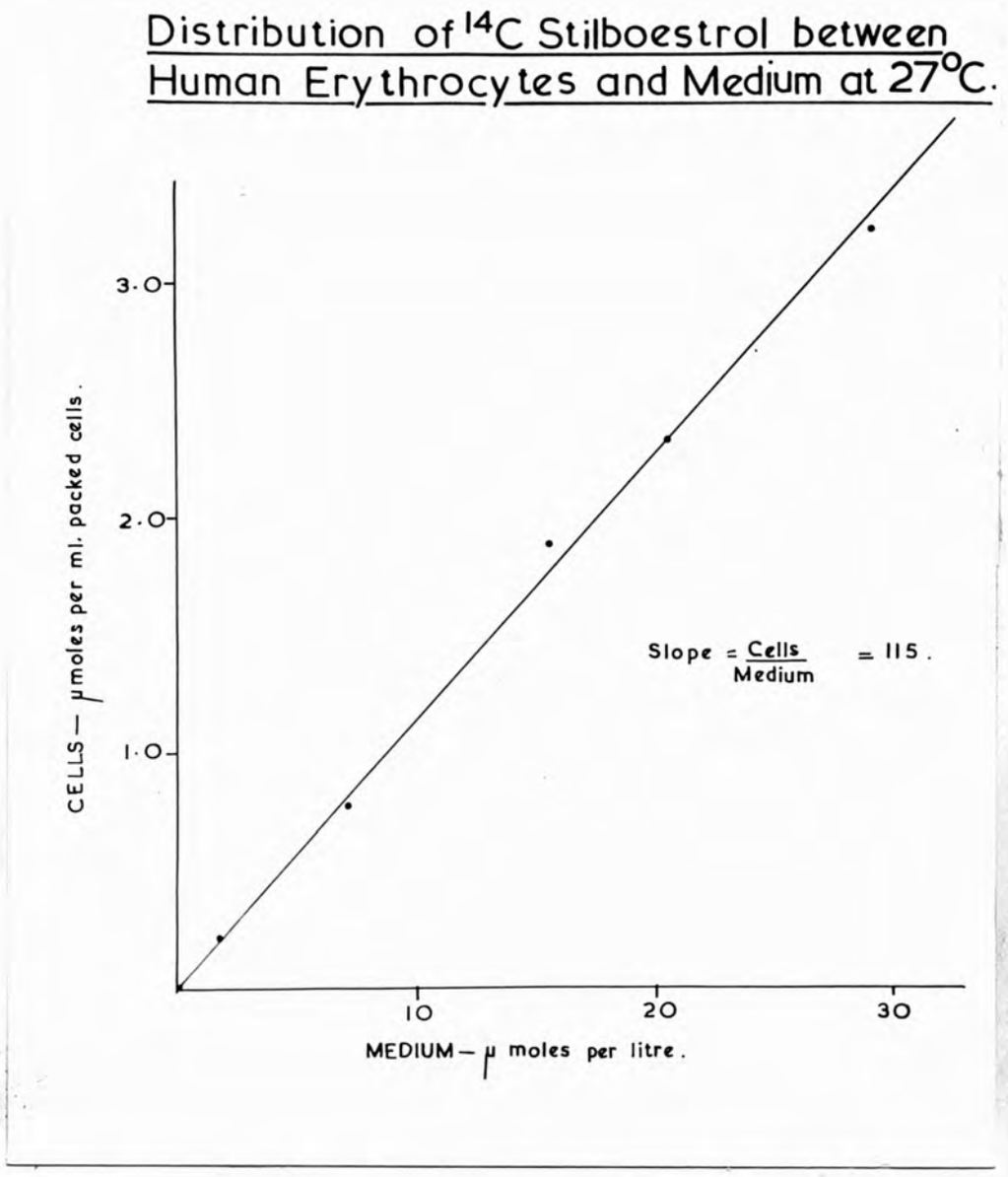


Fig. 7.7. Data plotted from distribution experiments showing relationship of stilboestrol concentration in medium to that in red cells. The figures for red cell concentration are calculated on the assumption that stilboestrol enters the red cell and freely distributes through the cell water.

Note. The figures for concentration in the medium are per litre of fluid and hence cell conctrations dhould be multiplied x 100 to make a direct comparison.

Haematocrit tubes were then filled to the 1.ml. mark with distilled water and the cells haemolysed. Two 0.05 ml. samples were taken for counting and 2 x 0.05 ml. into distilled water for haemoglobin determination. Twenty minutes after the commencement of incubation with stilboestrol a second series of determinations were made (series b). The results are summarised below. The values for uptake by the cells are calculated on the assumption that stilboestrol is taken up and distributed through the whole cell.

Tube	N Moles Stlb per ml. cells	N Moles Stlb per ml. Medium	Distribution Ratio of Stlb-cells/ Medium.
1a + 1b	207	1.64	126
2a + 2b	775	7.03	110
3a + 3b	1,886	15.45	122
4a + 4b	2,332	20.30	115
5a + 5b	3,015	26.73	113

There is a direct linear relationship between stilboestrol concentration in the cell and concentration in the medium. Thus uptake of stilboestrol by the cell seems to be a reflection of the distribution between the cell constituents and the saline medium. Since stilboestrol concentrates within the cell it appears that it would not be so useful for labelling the carrier site as hoped.

Results of Uptake and Extraction Studies Using ^{14}C-labelling.

CHAPTER 8.

Lipid extracts of human and sheep erythrocytes incubated with ^{14}C-DNFB and ^{14}C-NEM.

Introduction.

The foremost consideration in the problem of carrier transport is the chemical nature of the proposed membrane component with which glucose reacts. At present only a negative statement can be made. Inorganic phosphorus as was assumed in the phosphorylation theory of sugar absorption from intestine and kidney tubules must be discounted. Rosenberg, Vestergaard-Bogind and Wilbrandt (1956), have shown that neither glucose-6-phosphate nor glucose-1-phosphate nor any of the other hexose monophosphate esters which they tested were capable of penetrating the red cell membrane.

A prior requisite for the possible isolation of the proposed phospholipid membrane carrier is its stable marking. This has been illustrated in a preliminary paper by Stein. Two reagents DNFB and phenyl isothiocyanate were found to react with the site implicated in glycerol transfer, although in quite another way than the normal substrate and seemingly with only a portion of the site. The experiments with DNFB already described also appear to indicate that labelling of the component is possible. These various experiments appeared promising enough to encourage isolation experiments.

Lipid Extracts of Human Red Cells Incubated with ^{14}C-DNFB.

Time expired cells were used. A series of glucose exits and entries were followed in these cells and the parameters of transfer seemed to be normal. This gives a further indication that metabolism

is not directly involved in glucose transport in the human erythrocyte as in these cells the ATP supplies are depleted and much Na^+ has leaked into them.

The cells were inhibited by incubation with DNFB in the same manner as for uptake experiments. In 100 ml. glucose containing saline 50 ml. packed cells were suspended and allowed to equilibrate at 37°C for thirty minutes. They were transferred to a bath at 33°C and 133.5 ml. glucose-saline added containing 16.5 ml. alcoholic ^{14}C DNFB (as used in uptake experiments) so that a final concentration of inhibitor of 1.4 mM was achieved. The mixture was spun and the 48 ml. cells recovered were added slowly to 480 ml. butanol with constant stirring and left to extract overnight at 4°C under an atmosphere of nitrogen. No aqueous layer separated out and the total extract was filtered.

To the remaining solids were added 100 ml. methanol, the whole shaken well and filtered. The residue was extracted with a mixture of 190 ml. ethanol, 20 ml. concentrated HCl and 30 ml. water The mixture was shaken and allowed to stand at room temperature for two hours and filtered: 250 ml. of 0.9% NaCl were added to the remaining solids, shaken and the mixture allowed to stand at 4°C. Samples of 0.2 ml. of each of the extracts were taken and the total counts in each extract calculated.

The percentage recovery of radioactivity in each extract was: butanol 2.26%, methanol 0.28%, acid alcohol 5.58% and remainder 91.9% The remaining solid residue was largely haemoglobin. The butanol extract contained the equilivent of 10.1 umoles labelled DNFB/ 50

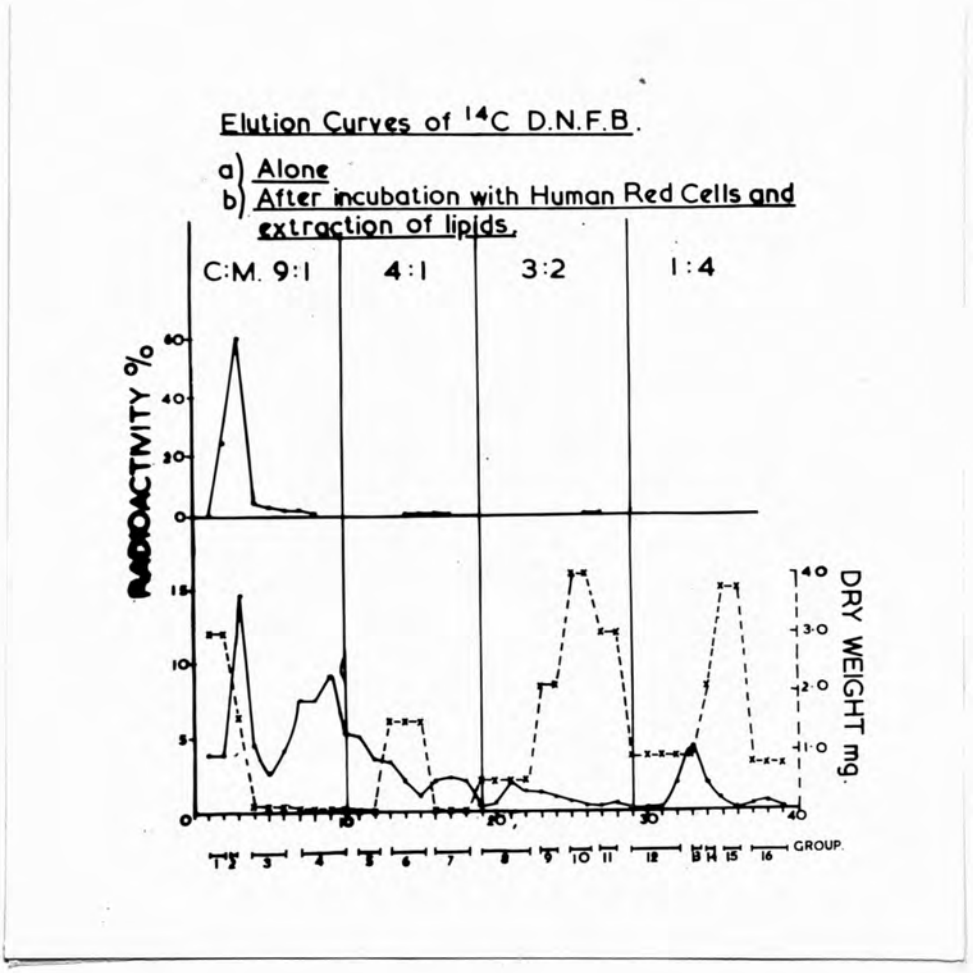


Fig. 8.1. - Pattern of elution of ^{14}C-DNFB from silicic acid column a) alone and b) after incubation with human erythrocytes, and extraction of lipids with n-butanol - 50 mg of extract eluted from a column of 5 g silicic acid and 2.5 g Hyflo-supercel with mixtures of chloroform:methanol. The fractions were pooled in groups as indicated and further examined by thin layer chromatography. Broken line indicates weight recovered in each group of fractions.

ml. red cells. Expressed in molecules of DNFB this is 10 - 20 x 10^6 per red cell which is a more likely estimate of the number of carrier sites available than obtained in the uptake experiments. Even so the amount of DNFB recovered in the lipid extracts could be up to twenty times more than enough to account for the glucose transfer sites, if these had in fact been recovered during the course of the extraction.

The butanol extract was taken to near dryness, during which procedure two thirds of the radioactivity was lost, probably largely by evaporation suggesting that much of the DNFB was not firmly combined. The dried extract was taken up in chloroform and the insoluble residue in carbon tetrachloride. Of the 138 mg of chloroform soluble extract obtained (with a total radioactivity of 51,200 counts/minute representing 6 μmoles) 50 mg were fractionated with four elution mixtures on silicic acid column containing 5 g silicic acid. Samples were taken for radioactive counts. The fractions were pooled to give measureable dry weights and evaporated under reduced pressure at below 37°C. The pooled fractions were examined first by infra-red spectroscopy and then by thin layer chromatography. Those spots with at least 700 cts/min were scanned for radioactivity.

Fig. 8.1. shows the elution pattern of radioactivity (solid line) and weight (broken line) of lipids of DNFB treated red cells. The upper record represents the elution of pattern of DNFB alone. It appears that DNFB is not absorbed, or only to a very small

degree and travels with the early part of the first eluent in the column and with the solvent front on the thin layer plates. From the results of the thin layer chromatography and the infra-red spectroscopy it was only possible to identify the major components of the peaks eluted. Complete separation of the components of the mixture was not achieved on the column, but the general pattern was as follows:-

Groups 1, 2 and 3 (as marked below on the diagram) contained neutral lipids and cholesterol and fast moving phosphorus containing components, probably phosphorus containing degradation products. Groups 5, 6 and 7 contained largely phosphatidyl ethanolamine. Groups 8 and 9 appeared to be the major phosphatidyl inositol containing groups. Phosphatidyl serine appeared chiefly in 7, 8 and 9, whilst lecithin was found in 10 and 11 and in 12 and 13 which were pooled before running on thin layer chromatography. Sphingomyelin composed the major portion of groups 15 and 16 where lysolecithin was also found.

Radioactivity was associated with several of the spots separated by thin layer chromatography. Groups 1 and 2 contained radioactivity found at the solvent front, which was probably free DNFB, but could have been associated with fast travelling lipids such as glycerides and sterols. A second small peak of radio-activity was found in group 2 with Rf 0.35 on thin layer plates. Radioactive peaks were also found near the origin in groups 3 and 5 and near the origin and in a position with Rf between 0.15 and 0.3

in 6 and 7. In 12 and 13 radioactivity was found associated with a non-phosphorus containing compound at 0.7 and with a ninhydrin staining spot at Rf 0.1. It is thus possible to obtain radioactive labelling which accompanies several lipid fractions during chromatography. No attempt was made at this point to identify the compounds associated with radioactivity.

Extracts of Human and Sheep Red Cells Treated with ^{14}C- DNFB.

Although it was found possible to label lipids with DNFB in these experiments it was not possible to determine which inhibitior complex if any was associated with glucose transfer. Results from extractions using human blood were, therefore, compared with results from a parallel experiment using sheep cells which do not exhibit the phenomenon of facilitated transfer. It was considered that the difference in labelling of lipids between the two experiments might give an indication as to which component is involved in glucose transfer. Fresh cells were used in the following experiments so that a more valid comparison could be made.

Packed sheep cells (30ml) were incubated with 76 mM glucose for a few minutes at 33°C and incubated for one hour at 33°C with a 1.4 mM DNFB solution containing a quantity of ^{14}C-DNFB and extracted with 300 ml. butanol. The percentage of radioactivity recovered in the butanol was 2.7 and of the 270 mg lipid obtained, which were taken up in chloroform, 50 mg were fractionated on a silicic acid column, samples being taken to estimate radioactive and phosphorus

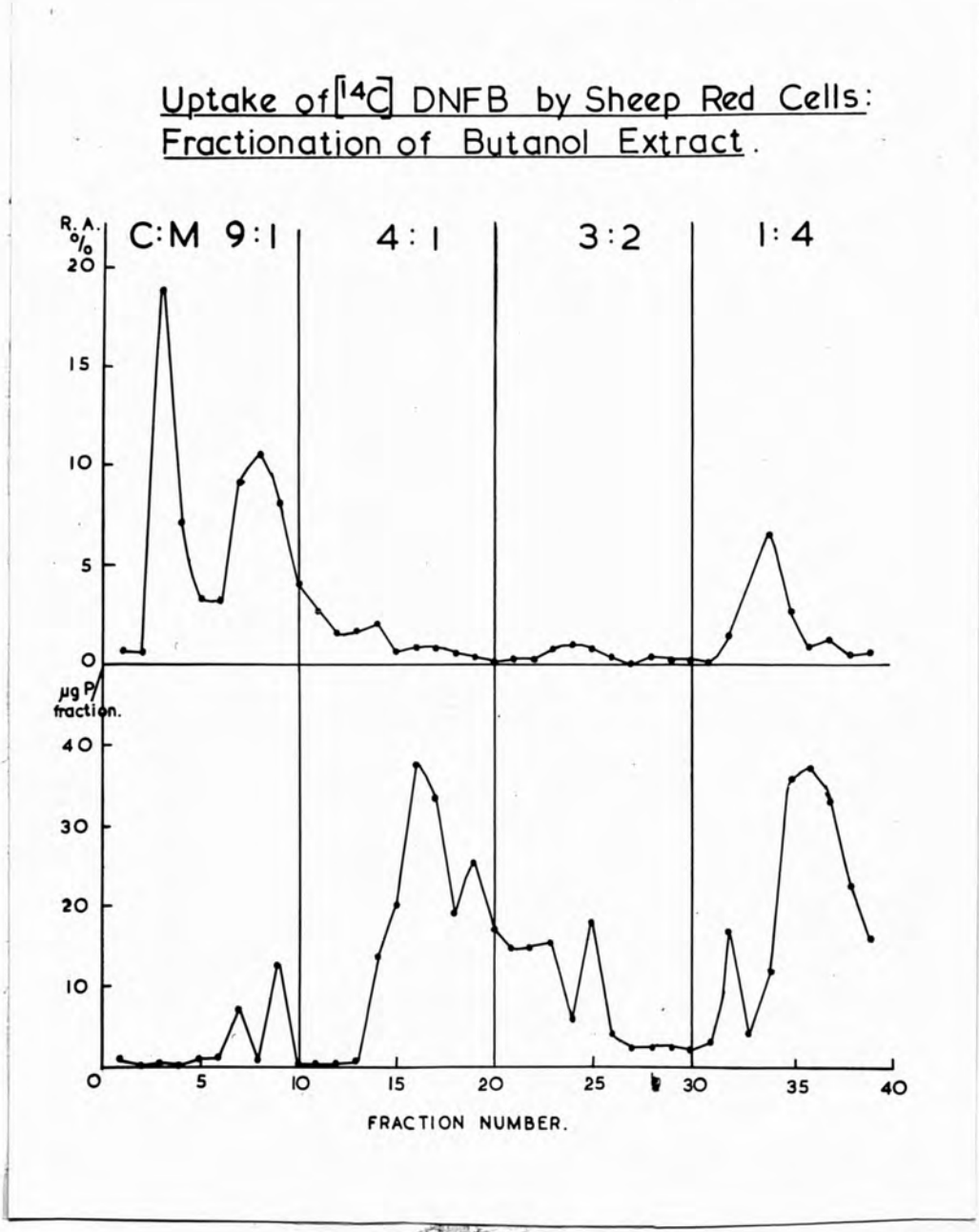


Fig. 8.2. Silicic acid column chromatography of lipids extracted with butanol from sheep red cells incubated with ^{14}C-DNFB.

1. Recovery of radioactivity, counts per fraction % of total.
2. Phosphorus content of fraction - µg P per fraction.

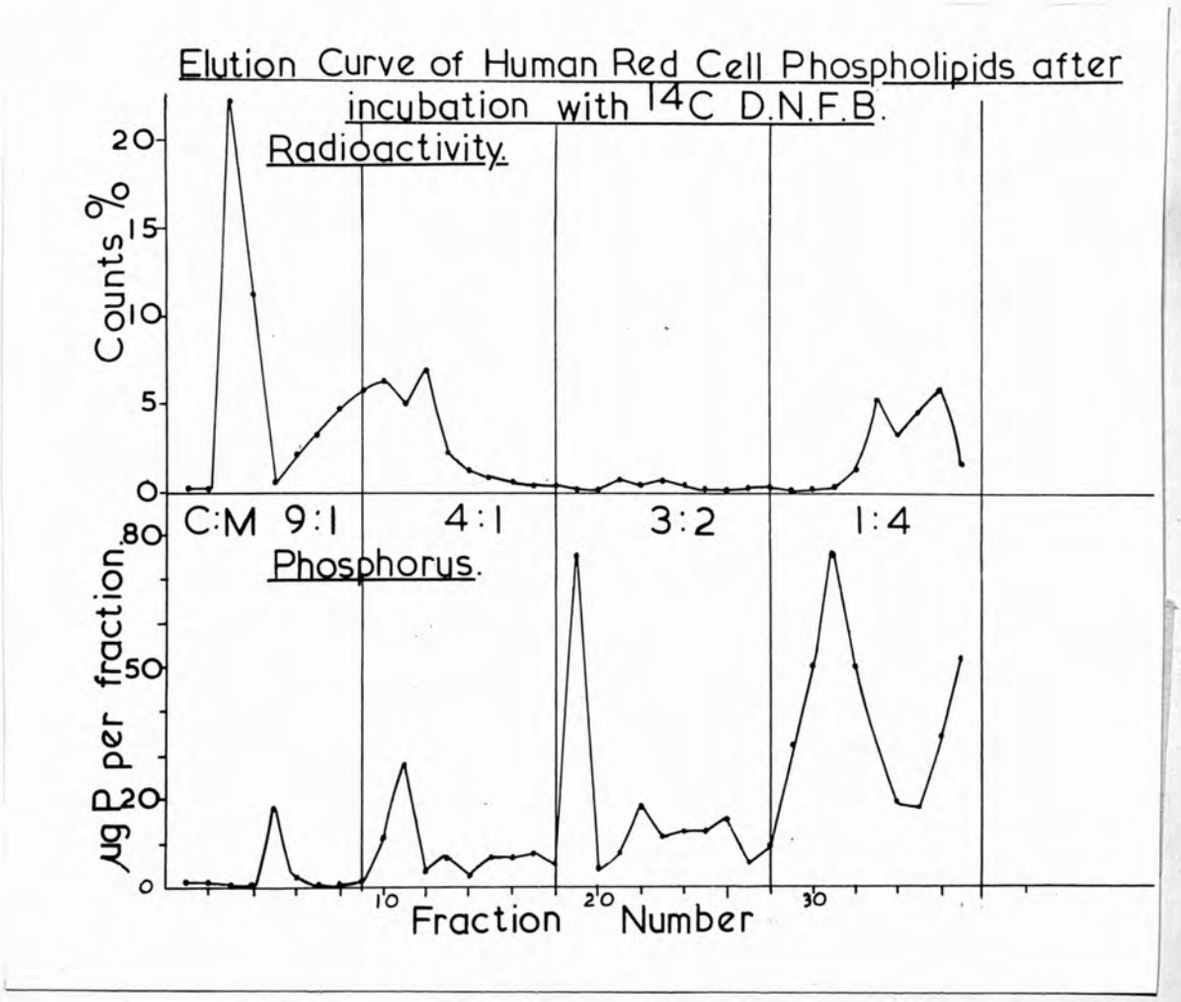


Fig. 8.3. Silicic acid column chromatography of lipids extracted with butanol from human red cells incubated with ^{14}C DNFB.

1. **Recovery of radioactivity counts per fraction % of total.**
2. **Phosphorus content of fraction - µg P per fraction.**

content. Fractions were also pooled to obtain dry weights.

Thirty millilitre of packed human red cells were similarly treated. The results of the experiments can be seen in figs 8.2. and 8.3. An example of the thin layer plates is seen in fig 8.4. stained with iodine and silver nitrate. Ninhydrin was not used as it was of little significance since DNFB had probably reacted with most free amino groups.

The distribution of lipids was similar to that in the previously described fractionation, except that sheep cells were found to contain more sphingomyelin and less lecithin than human cells. In both experiments a large early peak of radioactivity was found in fractions 3 and 4, the majority of which ran to the solvent front on thin layers and probably represents chiefly unbound DNFB, although with the sheep cells a slower moving component was also found which stained with iodine. The difference between the two experiments lies essentially in the second peak. With sheep cells ^{14}C labelling was found in fractions 7 to 9 which moved close to the solvent front associated with a phosphorus containing lipid. With the human cells the peak extended from fraction 9 to 13 and the radioactivity from the early part was associated with two slower moving lipids with the Rf of phosphatidyl ethanolamine. The spread of radioactivity is unlikely to be due to poorer separation on the column as the phosphate peaks separated discretely. In the extract of human red cells there was also a quantity of radioactivity associated with fractions 25 to 37 with similar Rf to that of

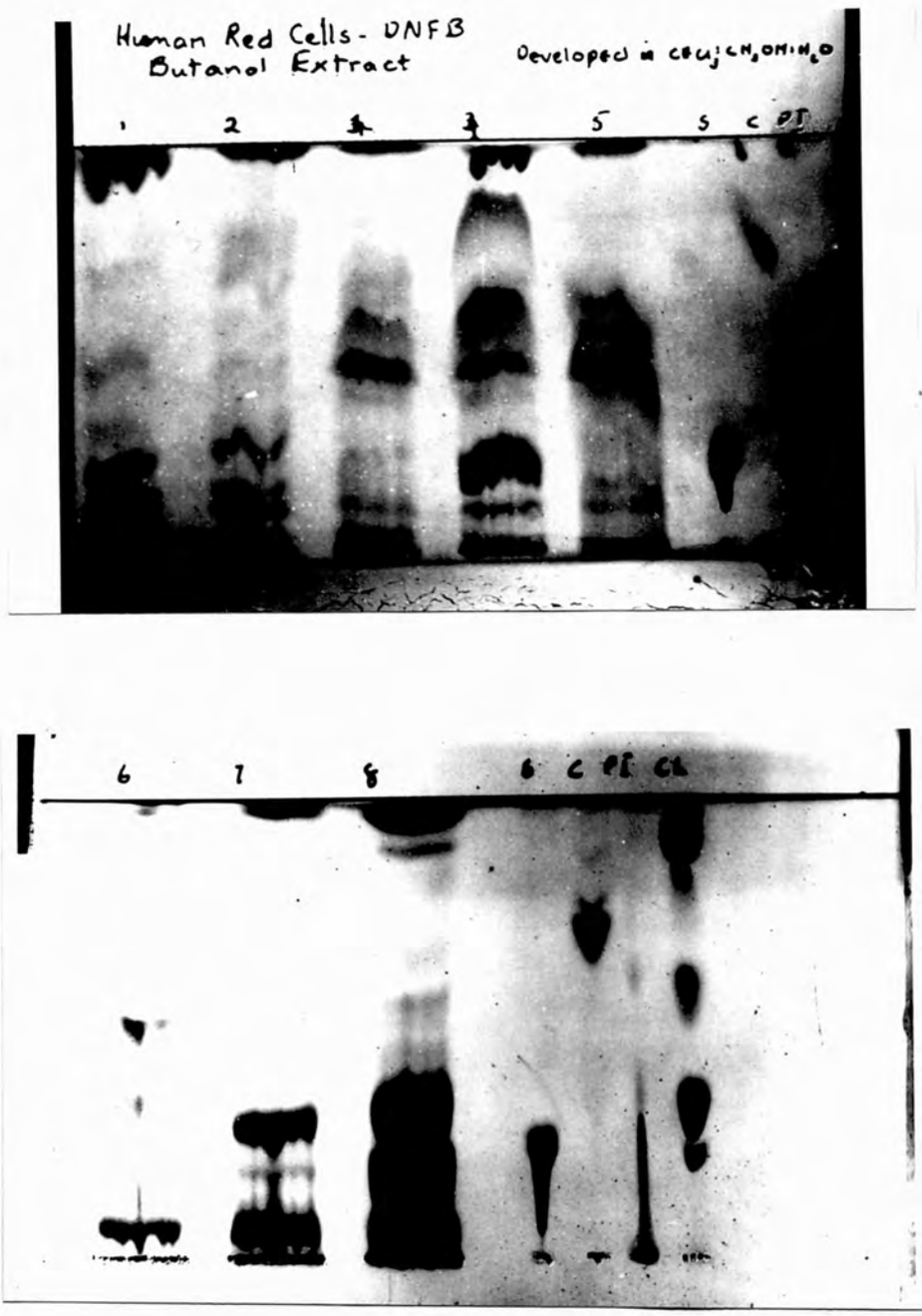
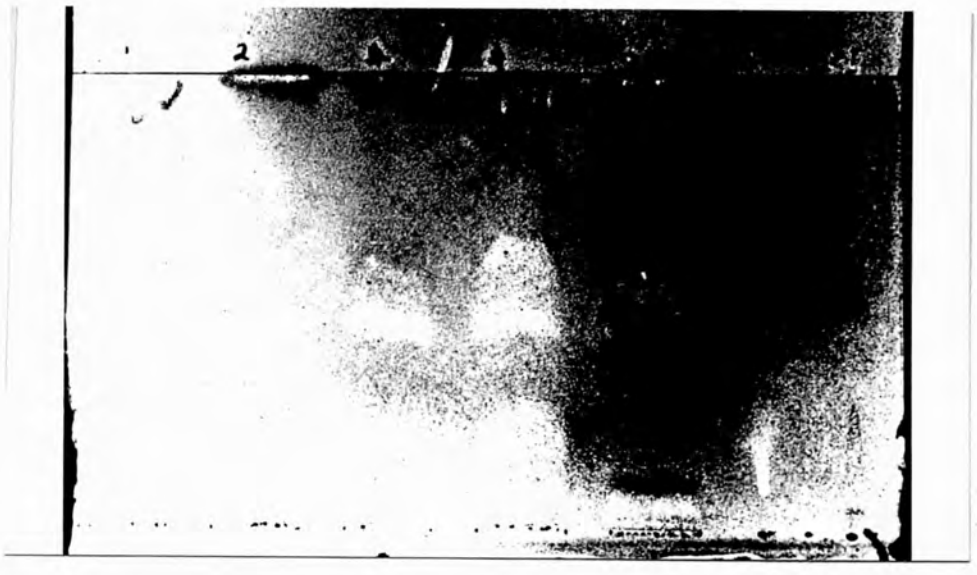


Fig. 8.4.a - Thin layer chromatograms of butanol extracts of human red cells treated with DNFB.

Plates developed as usual and stained with iodine.

Lipid markers S - sphingomyelin; C - cerebrosides; PI - phosphatidyl inositol; Cl - cardiolipin.



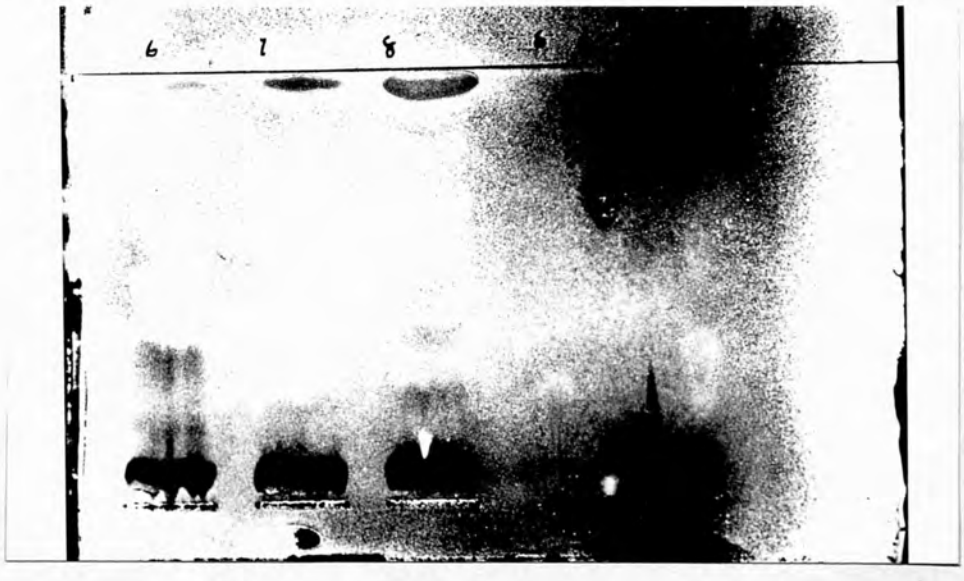


Fig 8.4.b - Thin layer chromatograms as in fig. 8.4.a, stained with silver nitrate.

sphingomyelin. The fixed DNFB does not correspond to any phosphate distribution in the early peak and in fractions 7, 8 and 9 (column 6) is present in greater molarity than the phosphate so that a non-phosphorus containing lipid is suggested.

From these results it appears that the fraction of phospholipids of greatest interest is that part eluted early in chloroform: methanol 4:1, associated with phosphatidyl ethanolamine. However the behaviour of the labelled compound on the column cannot be taken as a direct indication of the component involved as if DNFB had reacted with the head group of the lipid it would probably run differently.

An attempt, therefore, was made to identify the moiety with which DNFB had combined. The remainder of the human red cell extract which had been stored at 4°C was fractionated on a silicic acid column, the elution pattern being very similar to that already obtained. Fractions forming the three radioactive peaks were pooled, taken to dryness and taken up in approximately 0.1 ml. chloroform:methanol 4:1. The lipids were applied to the plates along a line of 3 cm. One spot of each sample was applied to the plate separately and ^{14}C-DNFB from the stock solution applied over them so that the position of free DNFB in the presence of lipids could be compared with the position of DNFB associated with lipids in the extract. The counts applied at the origin were determined and the plate was developed, stained with iodine vapour and scanned

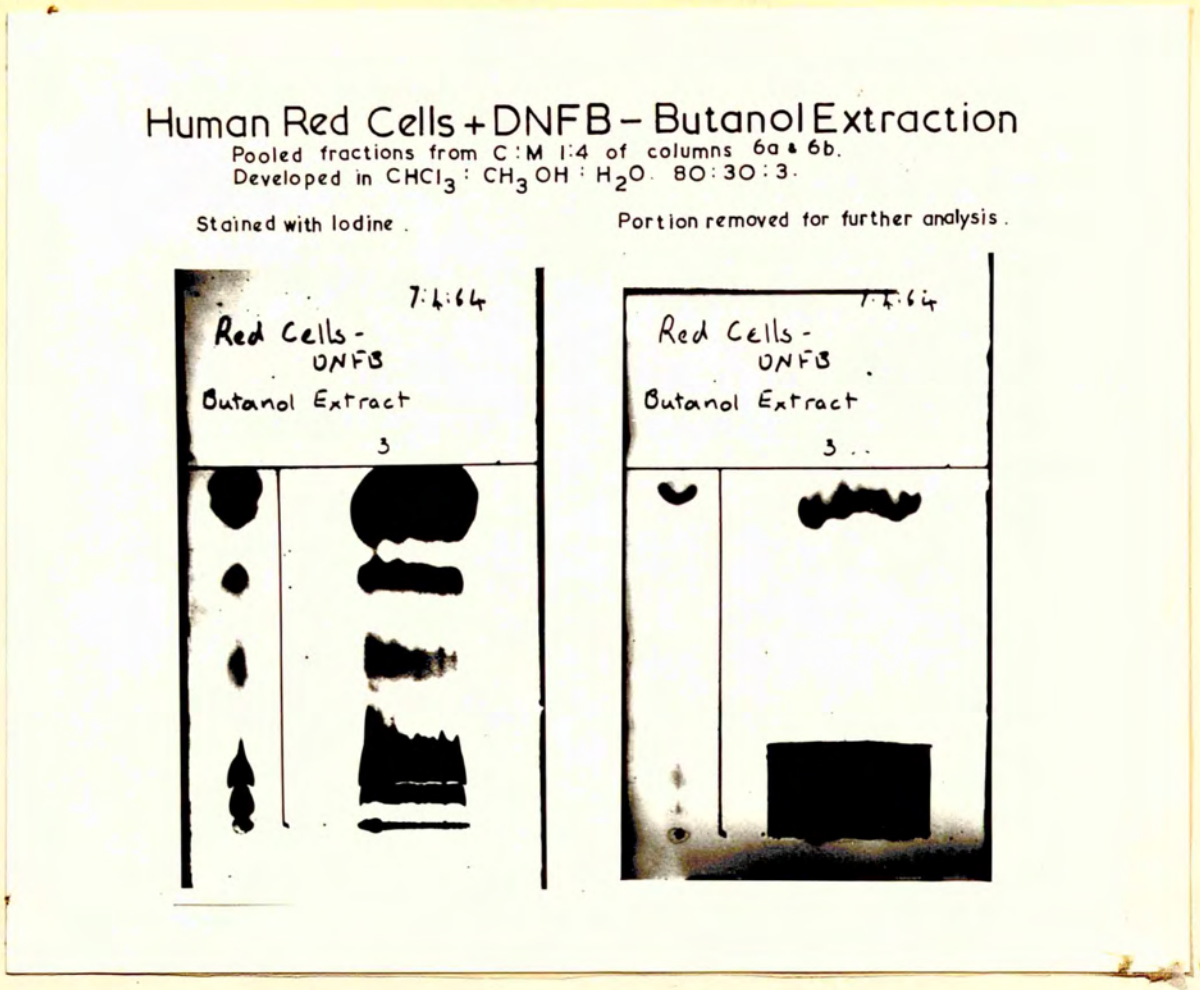


Fig. 8.5. - Thin layer chromatograms of chloroform soluble lipids in

(a) fractions 33-37 eluted from column 6a in chloroform:methanol 1:4.

Material applied over 3 cm band and plate developed in normal way stained with iodine.

Material on left represents portion over which free DNFB applied (see text)

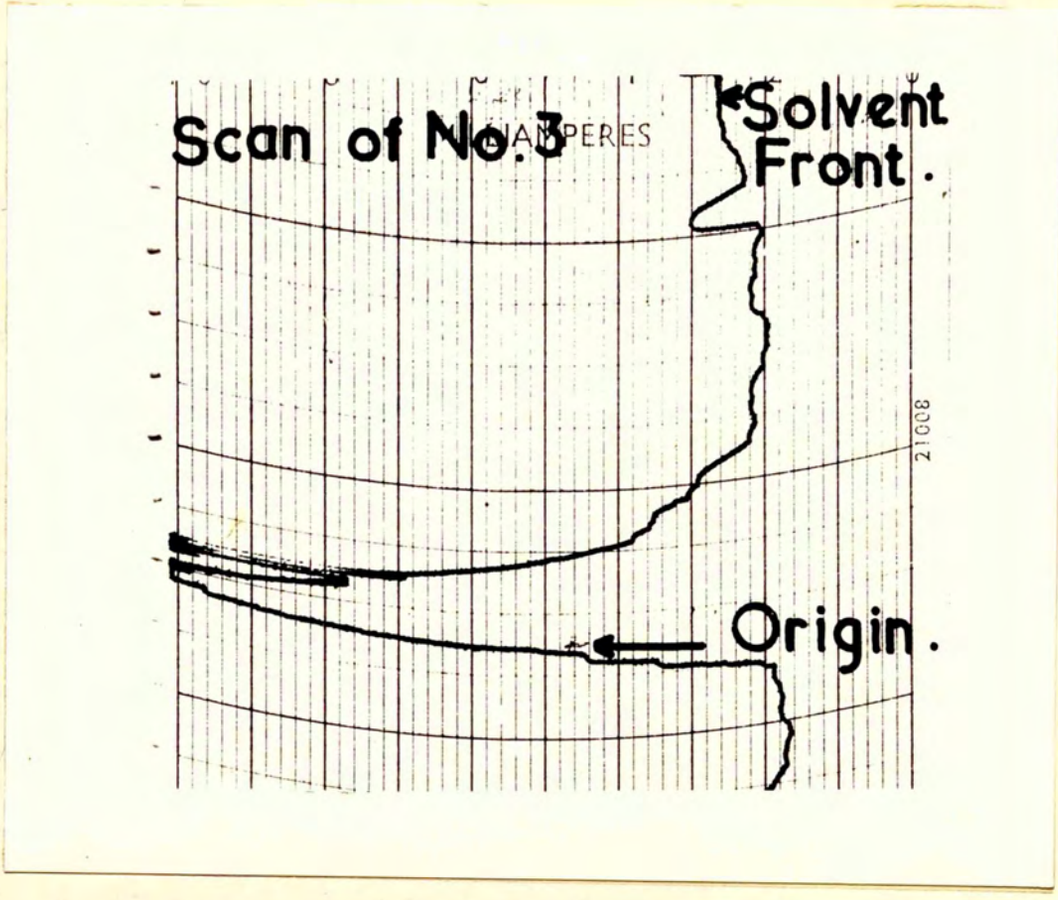


Fig. 8.5. (b) - Radioactive scan of sample 3.

Full scale deflection - 600 cts/min.

for radioactivity, although the position of DNP derivatives was obvious owing to the yellow colour. There were two radioactive peaks in the first two samples representing fractions 6 to 10 and 12 to 14 with Rfs of 0.6, 0.95 - 1.0 and 0.8, 0.95 - 1.0 respectively. The third sample representing fractions 33 to 37 contained one radioactive peak with Rf between 0.1 and 0.2 The silica was removed from the plates in the areas of radioactivity - see fig.8.5. and the lipids eluted as described in Chapter III. The material eluted from the solvent front in the first two samples was pooled so that four specimens of material eluted from plates were obtained:-

1. Material from solvent front of fractions 6 to 10 and 12 to 14.
2. Slow running material from fractions 6 to 10.
3. Slow running material from fractions 12 to 14.
4. Material from fractions 33 to 37.

Counts in the extracts were determined and the U.V spectrum recorded. Absorption maxima were obtained at 260 mμ and 248 mμ in specimen three. That for free DNFB is at 300 mμ.

The lipids eluted were hydrolysed for sixteen hours at 110°C with 5 N HCl and the hydrolysate shaken and extracted four times with 5 ml. peroxide-free ether. The ether extract was washed three times with water to remove any water soluble material. The aqueous layer remaining after the ether extraction was re-extracted with ethyl acetate and butanol and this extract was washed. The aqueous layer

separating from the butanol was retained as it would contain any free amino acids.

The ether soluble material of sample 1 was subjected to two dimensional chromatography to separate the DNP derivatives (as described in Chapter 3), the plate being scanned after development in each solvent. Two spots were obtained which could have been lysine DNP and possibly glycine DNP. The remaining samples were developed in one direction only with chloroform:tertiary amyl alcohol:acetic acid 70:30:3 with standards of ethanolamine DNP (Rf 0.56) phenylalanine-DNP (Rf 0.75) leucine DNP (Rf 0.82) tyrosine DNP (Rf 0.64) and glycine DNP The standards were prepared by treating amino acids with an equimolar solution of DNFB and extracting with acetone. No spots were visible in the samples, but elution of the silica between the origin and the solvent front in four sections for each specimen indicated that there was radioactivity in 2 and 3 between 0.5 and 0.75. The DNP derivatives were identified on the assumption that they were amino acid deravitives, but this need not have been the case. The water fraction from sample 1 contained material which gave a positive reaction with ninhydrin, but which could not be identified. Thus some indication was given of the material with which DNFB reacted but further characterisation was required.

Further Extraction of Human Red Cells Incubated with ^{14}C-DNFB.

The above experiment including incubation with inhibitor, extraction and isolation of lipids was repeated with time expired

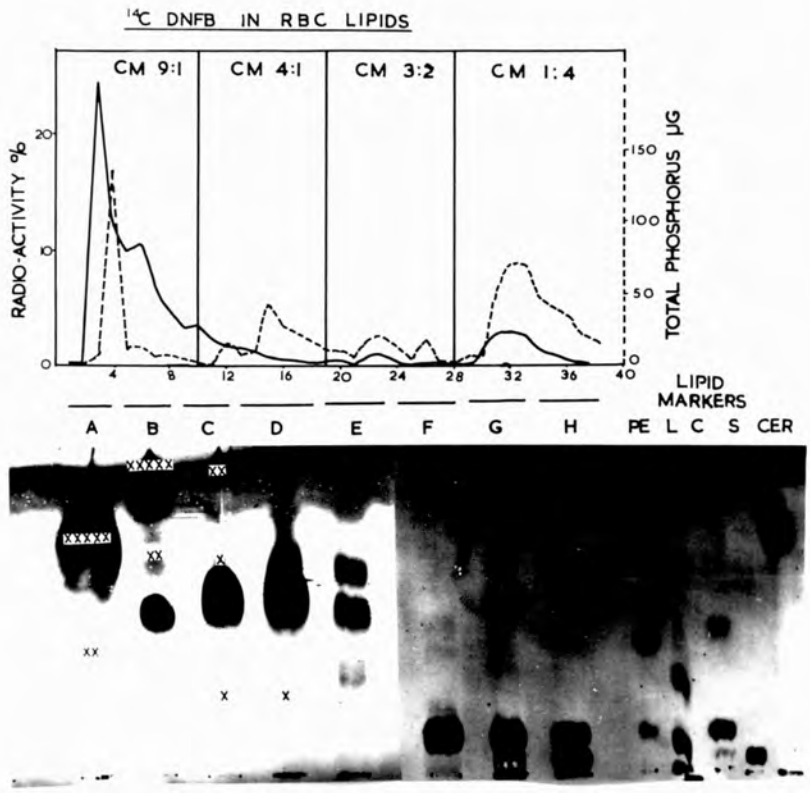


Fig. 8.6. Uptake of ^{14}C-DNFB by human red cell lipids.

Above elution curve of butanol extracted, chloroform soluble lipids- solid line represents percentage recovery of radioactivity - broken line, total phosphorus µg P per fraction. Fractions were pooled as indicated (A,B,C,D etc.) and further thin layer chromatography shown below. Spots visualised with iodine vapour. Lipid markers were

PE - synthetic cephalin (phosphatidly ethanolamine)

L - synthetic lecithin Card: cardiolipin antigen

S - sphingomyelin Cer: cerebroside.

cells and a higher concentration of DNFB. The percentage recovery of ^{14}C in butanol was 1.8% as opposed to 3.6% in the previous experiment and 0.45% in the chloroform soluble lipids as against 2.3% in the previous experiment. Thus the addition of six times the amount of ^{14}C-DNFB increased the uptake in chloroform soluble lipids by only 10%. A quantity of labelled material isolated in butanol was not soluble in chloroform. This chloroform insoluble portion was a pale yellow powder of fairly high radioactivity and gave positive protein reactions so that it could be proteolipid or protein in nature. Insufficient amounts were recovered for it to be investigated further.

The chloroform soluble material was fractionated on a silicic acid column, the elution curve being shown in fig.8.6. The fractions in each peak were pooled as shown (A,B,C,D etc.), taken down to dryness, redissolved in 0.1 ml. chloroform:methanol 4:1 and 5 ml. of this solution were applied to plates with standards and developed as shown in the lower half of the figure. The plates were stained with iodine, rhodamine-6-G and ninhydrin and scanned. The crosses indicate the position and relative strength of the radioactive spots. The remaining lipids of A,B,C, and D (pooled) and G were chromatographed in wide bands and the radioactive portions eluted as follows:-

Fraction.	Rf for Elution.	Thin Layer Chromatography Samples.
A	0.2 - 0.45	1a
	0.55- 0.95	1b

Fraction.	Rf for Elution.	Thin Layer Chromatography Samples.
B	0.3 - 0.6	2a
	0.7 - 1.0	2b
C + D	0.2 - 0.4	3a
	0.7 - 1.0	3b
G	0.02 - 0.08	7

The lipids were hydrolysed, extracted and taken up in acetone as described before. Fractions 1a, 1b, 2a, and 2b were applied to plates with standard DNP derivatives and separated in one direction only with two different solvent systems - chloroform:tertiary amyl alcohol:acetic acid 90:30:3 and benzene:pyridine:acetic acid 80:20:2. Both systems indicated that 1a and 1b contained 2,4-dinitrophenol and 1b, probably leucine DNP and 2b ethanolamine-DNP. The material in 2b was found to travel with ethanolamine-DNP. The fractions 3a, 3b, and 7 were run in one direction, using the second solvent mentioned, with standard DNP-amino acids. The material separated appeared to coincide with that of DNP-tyrosine.

The water fraction remaining after the extraction of DNP derivatives was evaporated near to dryness taken up several times in water and then evaporated down again to remove all traces of HCl, and finally taken up in N/10 HCl which was applied to plates. They were developed in chloroform:methanol:17% NH_4OH 2:2:1.

All four samples run (1a, 1b, 2a, 2b) appeared to contain amino acids -1a appeared to consist of serine, glycine, leucine, phenylalanine and possibly alanine, histidine and valine. Similar patterns were obtained for the other samples. Two way chromatograms were run on 2a and 2b, using chloroform:methanol:ammonia 2:2:1

in one direction and phenol:water 75:25 in the other direction. A spectrum of amino acids was obtained with both of the samples. They were tentatively identified as glycine, glutamine, lysine, arginine, alanine, valine, leucine and proline.

It is not known whether these lipo-amino acids were artefacts formed during extraction and isolation procedures. Wren (1960) has reported that these can be formed artificially, although he has obtained lipo-amino acids from blood which he believed existed "in vivo" and earlier Blass et al (1953) reported their presence in human erythrocytes and Turner (1957) in sheep erythrocytes.

These experiments involving the incubation of human erythrocytes with DNFB succeeded in that some lipids were labelled irreversibly and in quantities large enough for further analysis. Bound DNFB from early radioactive peaks appeared to consist to a large extent of DNP-ethanolamine, but if this is a DNP derivative of phosphatidyl ethanolamine then the DNP has clearly accelerated both the elution from the column and its rate of migration on thin layer plates. It is possible that DNFB attaches to a moiety other than amino acid, but this has not been determined.

Extracts of Erythrocytes Incubated with ^{14}C-NEM.

Similar experiments to those described above were carried out using ^{14}C-NEM. It was hoped that this might prove more specific than DNFB. Forty millilitre of packed cells (time expired) were equilibrated with 76 mM glucose and then incubated with 8 mM NEM.

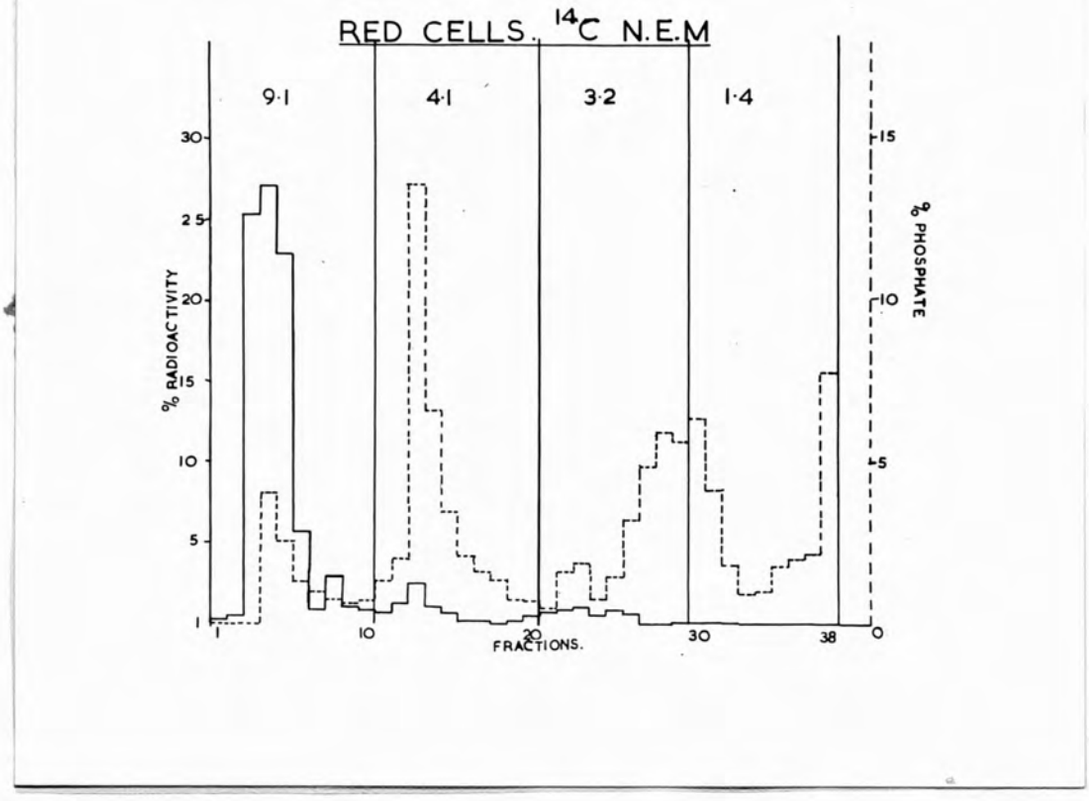


Fig. 8.7.- Pattern of elution of phospholipids and ^{14}C-NEM after extraction with butanol of human red cells incubated with 8 mM NEM.

Solid line % recovery of radioactivity per fraction.

Broken line line % recovery of phosphorus per fraction.

(From a stock solution containing 60 mg unlabelled NEM and 0.4 mg ^{14}C-NEM in 22 ml alcohol, 8 ml. were used to give this concentration). Samples of the incubation mixture were taken at various intervals to determine the percentage of inhibition of glucose exit. The cells were 92.5% inhibited at the end of one hour. They were washed and 30 ml. packed cells extracted with 400 ml. butanol. The majority of the radioactivity remained in the supernatant; only 7.4% remaining in the cells of which 1.3% was obtained in the butanol extract. The lipids obtained were taken to near dryness and dissolved in 1.2 ml. chloroform:methanol 9:1 and separated on a silicic acid column. The percentage of radioactivity obtained in the eluents is shown in fig. 8.7.

In some ways the elution pattern of ^{14}C-NEM is similar to that of DNFB, a large peak in the early fractions probably representing free material and smaller quantities with the second and third eluting solvents. The peak in the final solvent chloroform:methanol 4:1 found with DNFB is, however, absent. The spectrum of the material from fraction 3 was observed in ultra violet light. Surprisingly the characteristic absorbtion maximum at 300 mμ was absent.

Material from 3, 4 and 5 was further separated using thin layer chromatography. The material was applied in chloroform:methanol 9:1 along a 7 cm band. Lipid standards and 10 μl of the NEM stock solution were also applied. The plate was developed in chloroform:methanol: water 80:30:3 as usual and the plate scanned. Free NEM was found to

run to the solvent front. Radioactivity appeared at the solvent front associated with lipids and also at a position of Rf 0.2 to 0.3. Portions of silica between 0.15 and 0.35 and 0.8 and 1.0 were removed leaving a thin strip of lipid which was stained with iodine and ninhydrin. The fraction was found to contain mainly fast moving material and some with the Rf of cephalin, but ninhydrin negative. Ninhydrin staining is of no great significance as NEM may mask the amino group. The material was eluted from the silica with chloroform: methanol 9:1 and hydrolysed. The hydrolysate was found to contain small quantities of ninhydrin positive material, but no amino acids could be separated in chloroform:methanol:17% ammonia 2:2:1.

Labelling of a lipid fraction with NEM is, therefore, possible although little information as to the nature of the compound was obtained in this series of experiments.

Extracts of Human Erythrocyte Ghosts Incubated with ^{14}C-NEM.

Attention has already been drawn to the fact that only a small portion of material with which NEM reacts is found in the membrane. Quantities of labelling were, therefore, being lost, especially to haemoglobin. For this reason it was decided to repeat the experiments using erythrocyte ghosts. The ghosts, prepared by Miss D.A. Harris, seemed satisfactory when viewed under the phase contrast microscope. It was not known if the glucose transport mechanism was

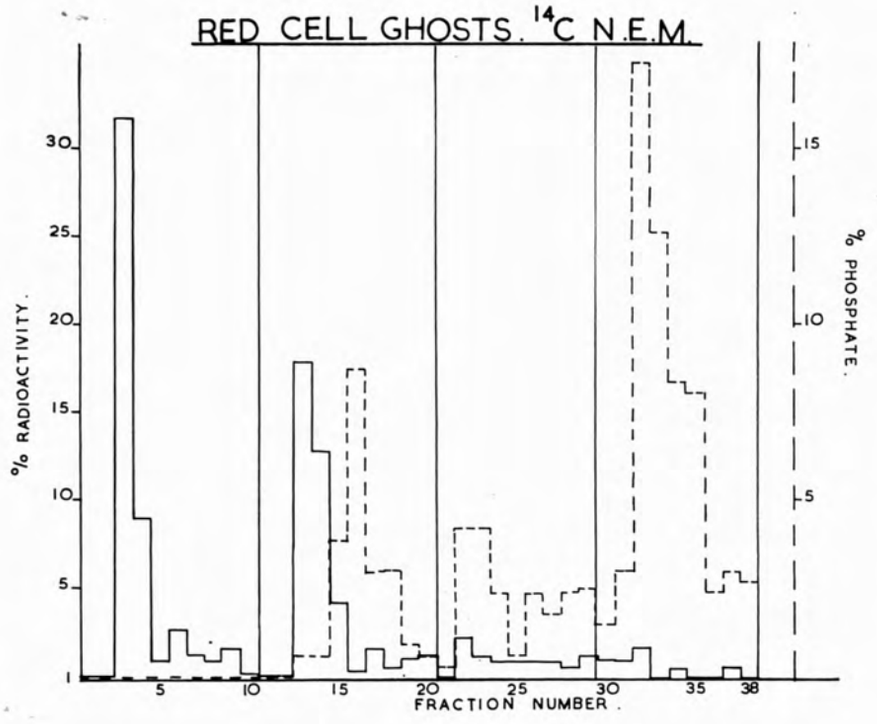


Fig. 8.8. - Pattern of elution of phospholipids and ^{14}C-NEM after extraction with butanol of human red cell ghosts incubated with NEM.

Solid line - % recovery of radioactivity per fraction.

Broken line - % recovery of phosphorus per fraction.
The eluting solvents were chloroform:methanol 9:1, 4:1, 3:2 and 1:4.

intact as the considerable reduction of haemoglobin prevented glucose movements from being followed in the photoelectric apparatus although this was attempted. Stein (1956) found that ghosts prepared by gentle hypotonic haemolysis have the ability to facilitate diffucion of glycerol and LeFevre (1961c demonstrated glucose transfer in the erythrocyte ghost. Many authors agree that the ghost, chemically at all events, is little changed from the intact erythrocyte membrane.

Unfortunately after these experiments were performed, notification was received from the Radio Chemical Centre, Amersham, that ^{14}C-NEM had proved unstable and only about 20% of the original material remained in their supply so that it was likely that decomposition had occurred in the solution stored in this laboratory. The results, however, are of some interest.

Incubation of 60 ml. ghosts was carried out for one hour at 33°C in 100 ml. glucose-saline containing 10 ml. alcoholic NEM solution, as used in the previous experiment. The solid material was spun off twice at 3,600 r.p.m. for half an hour. The first supernatant was taken and spun at 10,000 r.p.m. for 10 minutes. The 15 ml. material obtained from the three spinnings were extracted with 140 ml. butanol. Only 15% of the radioactivity added was recovered in the butanol so that an acid extraction was performed on the remaining material with no significant recovery of radioactivity. After the butanol had been evaporated 300 mg material were recovered which were disolved in 5 ml. chloroform:methanol 9:1. Of this solution

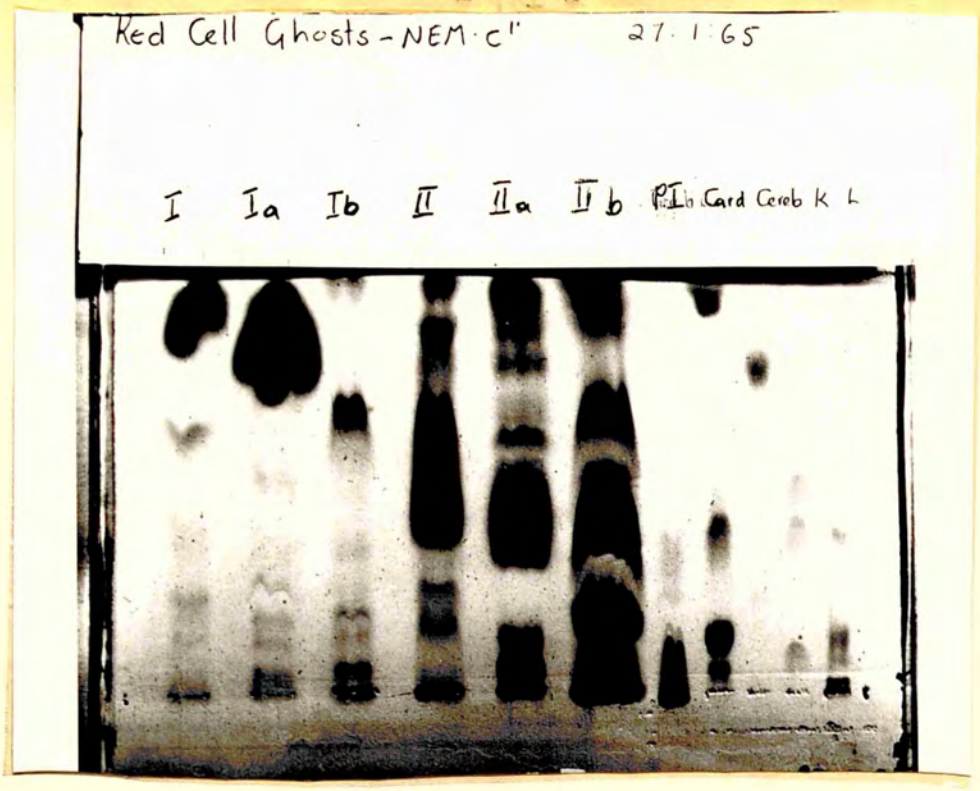


Fig. 8.9. (a).

T.L.C. of butanol extracts of red cell ghosts incubated with ^{14}C NEM.

Plates developed in usual way.

Fractions were obtained as follows:-

I. From fractions 3 and 4 of silicic acid column no. 12.

Ia From fractions 1 and 2 DEAE separation of material used in 1.

Ib. From fraction 17 DEAE separation of material as used in 1.

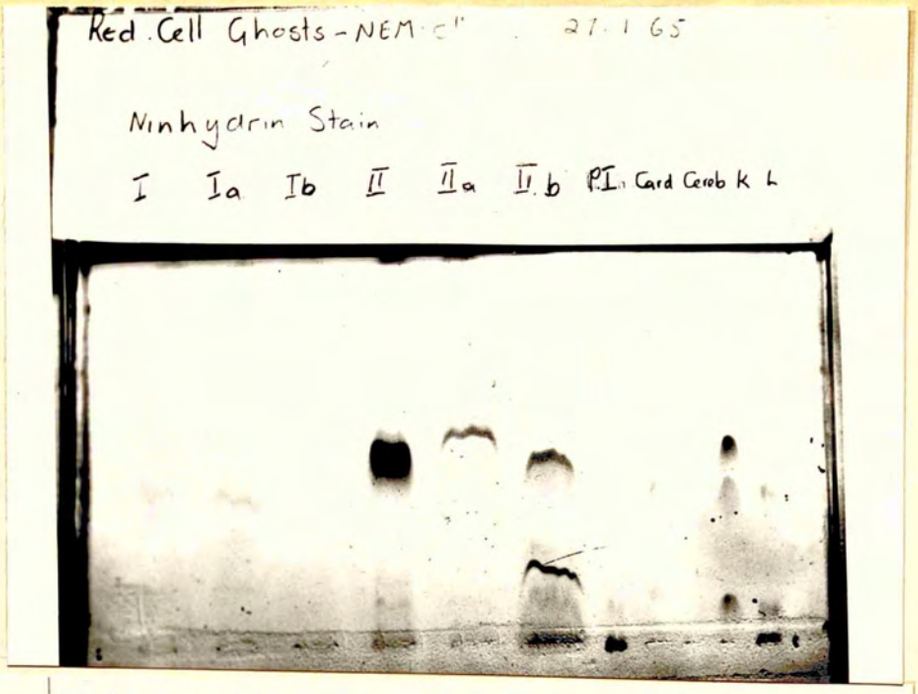
II. From fractions 13, 14, and 15 of silicic acid column no. 12.

IIa. From fractions 1 and 2 of DEAE separation of material used in II.

IIb. From fractions 15, 16 and 17 DEAE separation of material used in II.

Lipid markers - PI-phosphatidyl inositol; Card-cardiolipin; Cereb-cerebroside; K - cephalin; L - Lecithin.

(i)



(ii)

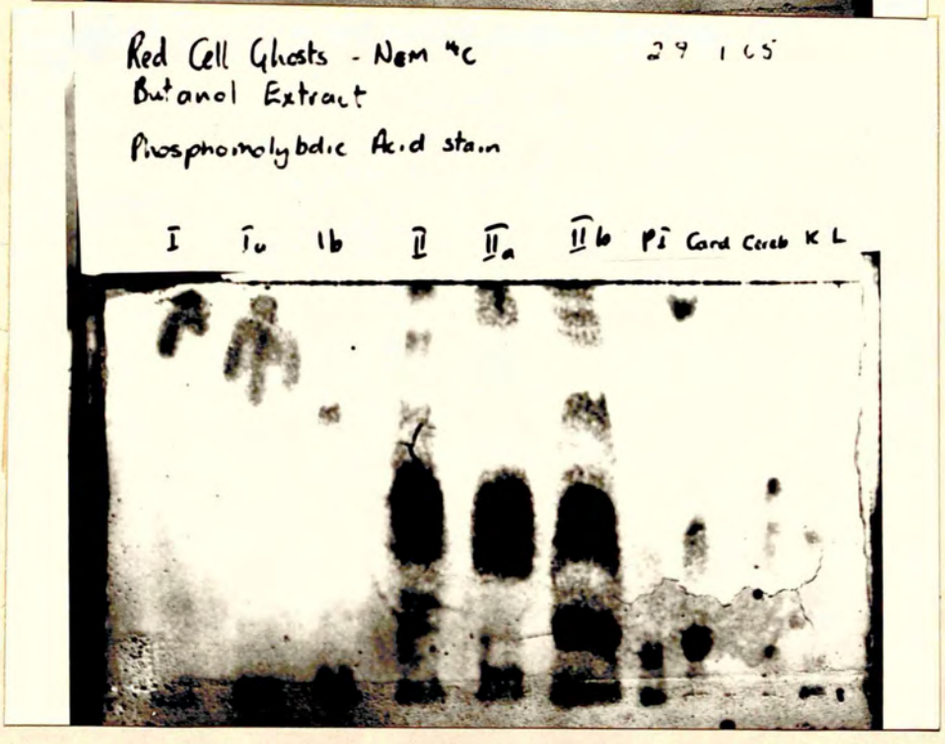


Fig 8.9. (b) - Plates as in fig. 8.9. (a) stained with

(i) Ninhydrin.

(ii) Phosphomolybdic acid.

2.5 ml. were separated on a silicic acid column (12.5 g silicic acid used). The quantities of radioactivity and phosphate recovered in each fraction is recorded in fig. 8.8. The elution pattern for ghosts is similar to that with erythrocytes, but the phosphate containing component in chloroform:methanol 9:1 is absent and the material obtained in the early part of chloroform:methanol 4:1 indicated a drop in the phosphatidyl ethanolamine content.

An initial large peak of radioactivity was found, but in contrast to the experiment using intact erythrocytes, a second quantity was eluted in chloroform:methanol 4:1.

Fractions 3 and 4; 13, 14 and 15 from the silicic acid column (no. 12) were pooled and further separated on DEAE cellulose as described in Chapter III. In both groups the majority of the ^{14}C-labelling was recovered in tubes 1 and 2, eluted in chloroform: methanol 9:1. That material from 3 and 4 gave a second small quantity of radioactive labelling in tube 17 (chloroform:glacial: acetic acid 3:1). The fractions containing ^{14}C-labelling were taken to dryness, dissolved in chloroform:methanol 4:1 and subjected to thin layer chromatography as shown in fig. 8.9. The fractions are as indicated in the legend to fig.8.9.

Fraction I. as would be expected, contained chiefly neutral lipids, especially cholesterol, although a slow running component appeared in Ib. II contained several lipids, but the chief of these was phosphatidyl ethanolamine as may be judged from the Rf value of the main component and the positive reaction with ninhydrin. Much

material was recovered in IIa and IIb. The greater quantity of phosphatidyl ethanolamine appears in IIbwhich also appeared to contain quantities of lecithin and phosphatidyl inositol. Rouser et al. (1963) found that phosphatidyl inositol appears chiefly in this fraction. Radioactivity in II was present at the solvent front, but that in II and IIb appeared in spots with Rf values of 0.4

Thus it appears possible to obtain NEM labelled material which is eluted in the same fractions as that labelled with DNFB. An attempt was made to identify the labelled material using the alkaline hydrolysis technique of Dawson.

The remaining material (that part of the extract not run on silicic acid column no. 12) was apread across the full width of a 20 cm plate which was developed in chloroform:methanol:water 80:30:3, scanned and the radioactive portion eluted, leaving a strip which was stained with iodine to confirm that lipids were present. Five hundred µg of phosphatidyl inositol, synthetic cephalin and lecithin were dissolved in 0.8 ml. methanol. To this were added 7.5 ml. ethanol, 0.65 ml. water and 0.2 ml. aqueous NaOH and the mixture incubated for 20 minutes at 37°C. The pH, which must be alkaline, was checked, 0.4 ml. ethyl formate added to neutralise the excess alkali and the whole incubated for another five minutes. The hydrolysate was evaporated to dryness in vacuo and the residue partitioned by thorough shaking with 1 ml. of the upper phase and 2 ml. of the lower phase of a mixture obtained by shaking together 9 ml. water, 6 ml. isobutanol and 12 ml. chloroform. The

emulsion was centrifuged at 3,000 r.p.m. for ten minutes and the aqueous supernatant removed as thoroughly as possible. The material eluted from the plate was treated in the same way, but double quantities of reagents were used.

The hydrolysates were run in phenol water (4:1:V/V):acetic acid:methanol 100:10:12. The unknown gave a pink spot, Rf0.34 associated with some radioactivity, but which did not correspond with any of the standards nor free NEM. Thus ^{14}C-NEM attaches to a moiety of the lipid molecule which may be removed by partial hydrolysis, although the NEM-lipid bond would appear to be resistant to mild hydrolysis. The NEM may affect the running of the phospholipid hydrolystate and so make its identification difficult.

Unfortunately it was not possible to pursue this promising lead as there was no further supply of a stable form of ^{14}C NEM available.

Results of uptake and extraction studies using ^{14}C-labelling.

CHAPTER 9.

Lipid extracts of erythrocytes incubated with phenolphthalein and erythrocyte ghosts incubated with ^{14}C-stilbeostrol.

Extracts of Erythrocytes and Erythrocyte "Ghosts" Incubated with Competitive Inhibitors.

Since experiments carried out in an attempt to isolate complexes of lipid and non-competitive inhibitors were fairly successful, it was thought that competitive inhibitors might also give stable labelling of a membrane component.

Extracts of Erythrocytes Incubated with Phenolphthalein.

A column was first run of phenolphthalein alone when all the material was recovered in fractions 3 and 4 eluted with chloroform: methanol (9:1). The compound was detected by adding 0.1 ml. sodium bicarbonate and 2.9 ml. distilled water to the fraction eluted and shaking. After thirty minutes all the pink colour was found in the aqueous layer and could be read on a spectrophotometer at 555 mμ, It was found that 0.25 mg phenolphthalein could be satisfactorily detected in this way. It is likely, however, that this is a greater quantity than would complex with lipid material.

For the extraction, 32.5 ml. time-expired cells, were shaken with 8 ml 0.2 mM phenolphthalein (21 mg phenolphthalein in 0.8 ml. absolute alcohol and 7.2 ml. saline buffer). After extraction with 325 ml. Butanol, 60 mg of material were obtained which were fractionated on a silicic acid column. Nearly all the phenolphthalein added could be accounted for in that material obtained in fractions 3, 4, and 5. Thus it appears that no permanent complex formed, although a small quantity may have gone undetected.

Extracts of Erythrocyte Ghosts Incubated with ^{14}C-Stilboestrol.

In the second series of experiments ^{14}C- stilboestrol was used. A 25 ml. stock solution was prepared containing 1.8 μmoles stilboestrol with a total activity of 45 μc. Two portions of 50 ml. of ghosts were treated with 10 ml. of this solution. One portion was extracted with 300 ml. ethyl acetate at room temperature for one hour and the other with 300 ml. of butanol. The extraction flasks were shaken for the first fifteen minutes and allowed to stand for the remaining forty-five minutes. The material remaining after the ethyl acetate extraction was re-extracted with chloroform and acid chloroform: methanol.

Ghosts Treated with Ethyl Acetate. The total radio activity in the 10 ml. stock solution added was 2,400,000 counts/min so that the recovery of material after extraction with ethyl acetate was as follows:-

Solvent	R.A. Cts/min	Percent Recovered
Ethyl Acetate	545,650	22.7
Chloroform	961,020	40
Aqueous layer separated from chloroform	5,928	0.26
Acid chloroform methanol	95,280	4.0
Upper phase after acid chloroform extraction	1,710	0.07
Solid material at interface	8,000	0.3

If stilboestrol complexed with lipid soluble material it could be present in the ethyl acetate, the chloroform or the acid chloroform

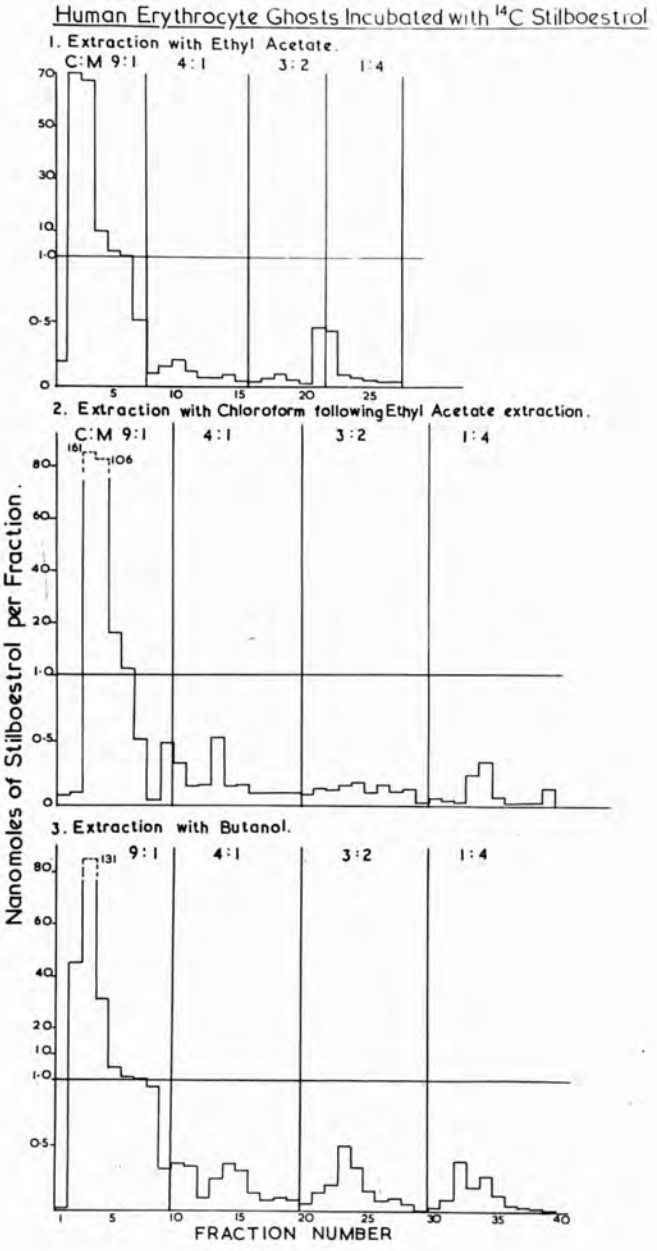


Fig. 9.1.

Chromatograms of lipids extracted from red cell ghosts incubated with ^{14}C-stilboestrol (cols. 36, 37, and 39). Extractions were carried out with 1) ethyl acetate followed by 2) chloroform & with 3) butanol alone. Column 36 - 40 mg of extract (1) eluted from a column of 4 g silicic acid and 2 g Hyflosupercel with mixtures of chloroform: methanol. Columns 37 and 39 were each 100 mg extracts of 2) and 3) respectively eluted from column of 10 g silicic acid and 5 g Hyflo-supercel with mixtures of chloroform:methanol.

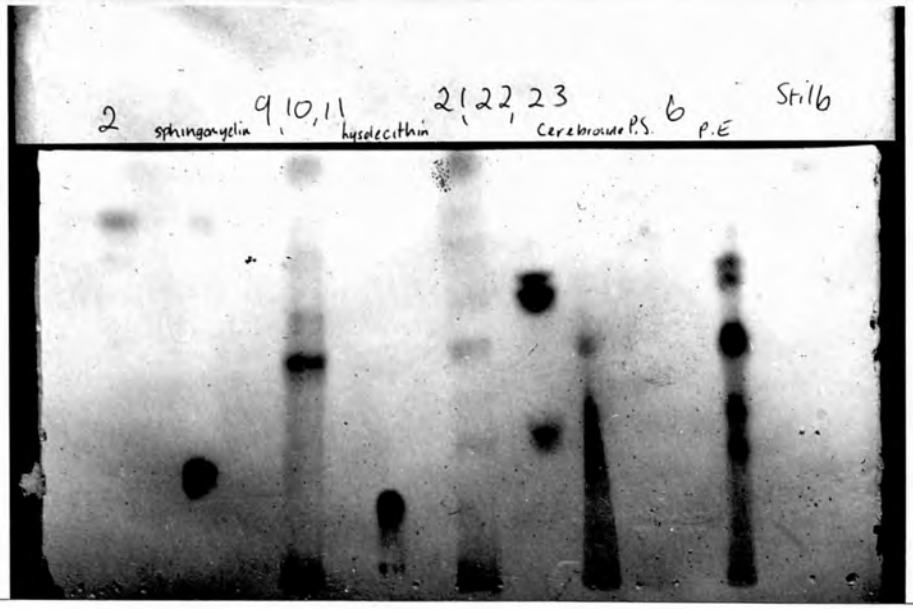
methanol as a complex of 20,000 cts. (8 nmoles) for as few as 10,000 molecules per cell, assuming that the complex is not broken down. This suggests that the activity in the acid chloroform:methanol is of little or no interest.

Recovery of lipid in ethyl acetate was very low - some 40 mg Separation was carried out on 4 g silicic acid and 40 ml. of each eluting solvent used (fig. 9.1.). The peaks of radioactivity were obtained in tubes 2 to 6, 9 to 11 and 21 to 23. Fractions 2, 9 to 11 and 21 to 23 were chromatographed on thin layer as shown in fig. 9.2. Fraction 2 contained fast moving lipids, 9, 10 and 11 some fast running material and phosphatidyl serine, while 21 to 23 and 6 contained very little lipid. Free stilboestrol appeared with a Rf value of 0.81. The radioactivity trailed slightly on these plates, but the peaks were at 0.8, 0.8, 0.8 and 0.77 in fractions 2, 6, 9 to 11 and 21 to 23 respectively indicating free stilboestrol.

Separation of material from the chloroform extract gave the elution pattern shown in figure 9.1. Besides the free stilboestrol peak at fractions 3 and 4, two small peaks were found in fractions 9 and 10 and 13 to 15. The material from these fractions was pooled and chromatographed on thin layers, but all the radio-activity appeared ~~appeared~~ in the position of free stilboestrol except for a single peak of Rf 0.64 in fractions 13,14 and 15.

A column run of the material obtained in acid chloroform methanol

a)



b)

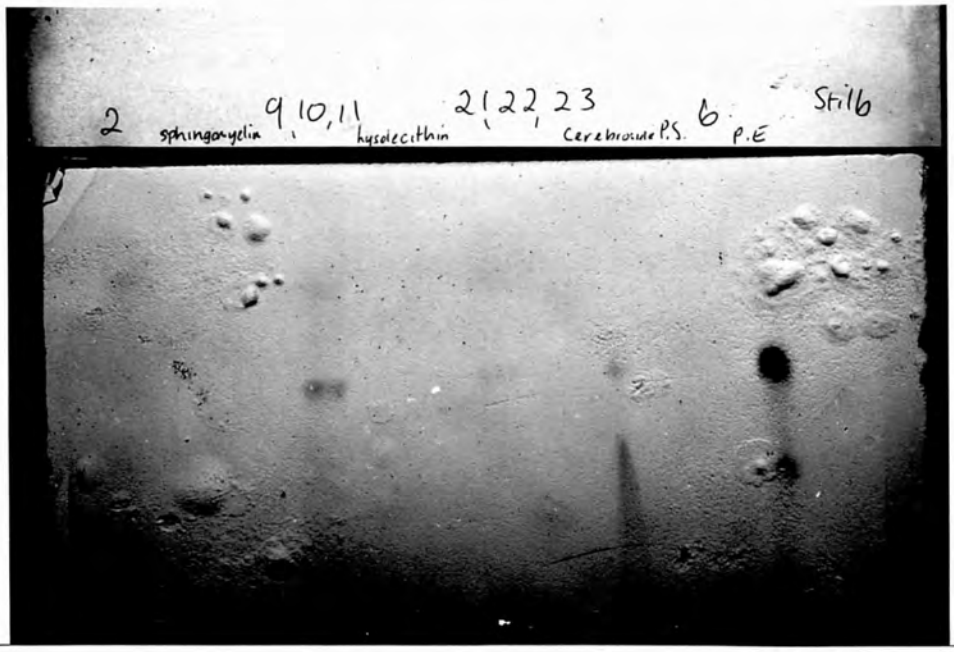
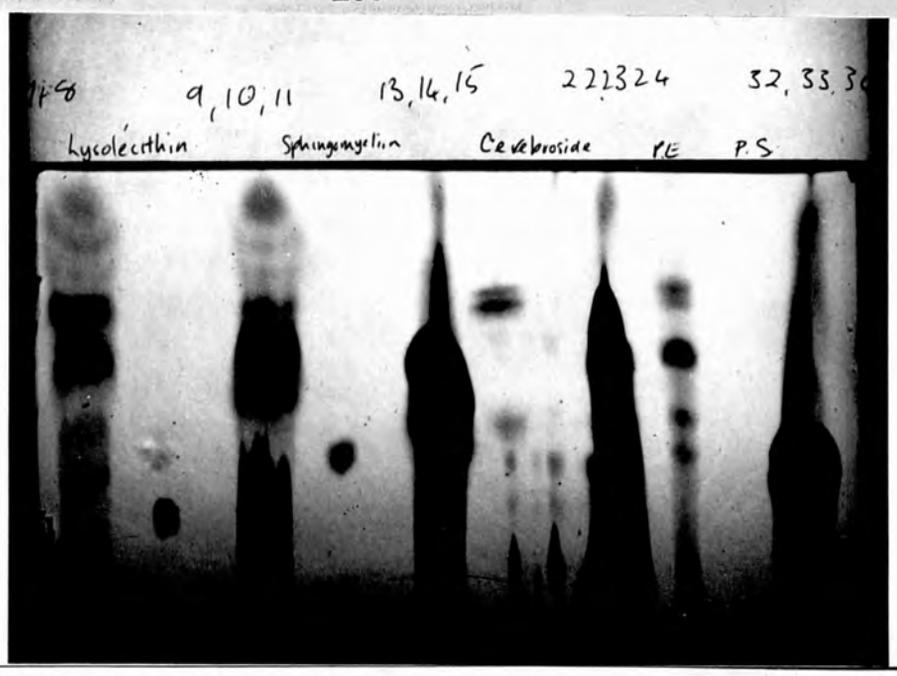


Fig. 9.2. - Thin layer chromatograms of lipids extracted with ethyl acetate from human erythrocytes incubated with ^{14}C-stilboestrol. Plates developed in usual manner and stained with

a) iodine. b) ninhydrin.

Fractions from silicic acid column (no 36) run as indicated. Lipid markers as shown and P.S - phosphatidyl serine. P.E.- Phosphatidyl ethanolamine.

a)



b)

Fig. 9.3. - Thin layer chromatograms of lipids extracted with butanol from human erythrocytes incubated with ^{14}C stilboestrol. Plates developed in usual manner and stained with

a) iodine b) ninhydrin

Fractions from silicic acid column (no. 39) run as indicated. Lipid markers as shown and P.E. - phosphatidyl ethanolmine and P.S. phosphatidyl serine.

yielded only free stilboestrol.

Ghosts Treated with Butanol. The recovery of radioactivity in the butanol was 875,100 counts/min representing 36.5% of the activity added. Separation of the butanol extract on silicic acid column gave a large early peak characteristic of free stilboestrol and five small peaks (fig. 9.1).

Peak Number.	Fractions.	R.A. cts/min.
1	7, 8	5,000
2	9, 10, 11	2,500
3	13, 14, 15	2,300
4	22, 23, 24	2,500
5	32, 33, 34	2,000

The peaks were small and likely to represent material held back by the extra lipids present in the butanol extract. Those of 2, 3, and 5 seem to have analogous peaks in the chloroform extract but the shoulder at 1 and peak at 4 were much smaller in the chloroform extract probably because of the preceeding ethyl acetate extraction.

Fig 9.3 gives an idea of the separation on thin layer plates. Unfortunately the plates had to be overloaded for sufficient radioactivity to be present for scanning. While the majority of radioactivity material in 1 and 2 ran with the Rf of free stilboestrol, there was a spread of radioactivity in the remaining samples, probably due in a significant part to streaking of the lipids. The radioactivity as a whole, however, did not appear much earlier with a

maximum at Rf 0.65 as opposed to 0.78 to 0.8 for free stilboestrol, and plateaux extending from 0.45 to 0.5 in 3, and 0.45 to 0.65 in 4 and 5. Some of this radioactivity may have been in the complex form.

The experiments were thus inconclusive. In view, however, of the experiments indicating that the action of stilboestrol may in part be due to preferential solubility in the membrane, this material may not be the most suitable with which to work. Polyphloretin phosphate which does not penetrate the cell would perhaps prove more useful.

Results of Uptake and Extraction Studies using ^{14}C-labelling.

CHAPTER 10.

Lipid extracts of human erythrocytes and human and sheep erythrocyte ghosts incubated with ^{14}C-glucose.

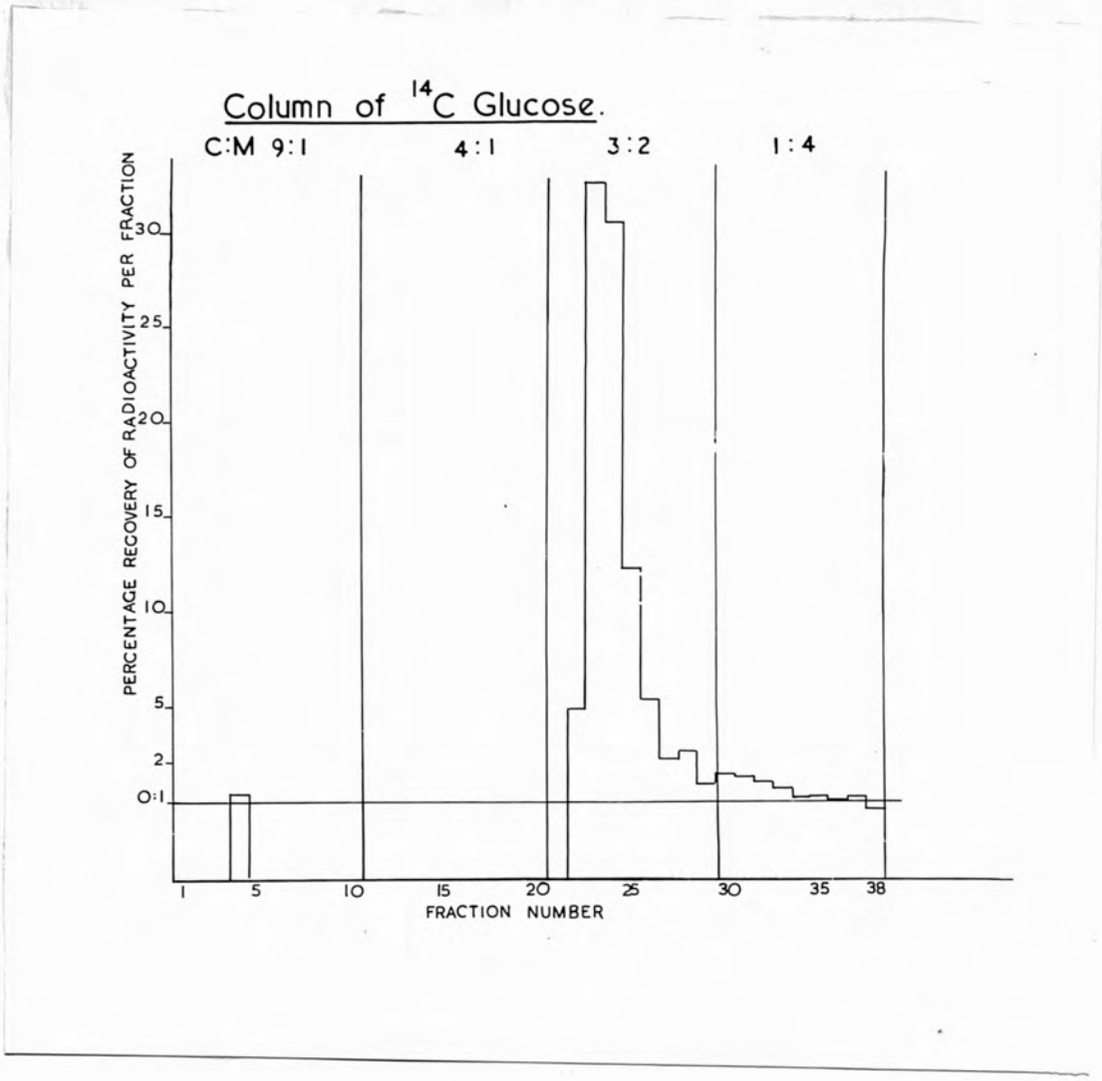


Fig. 10.1. (a) - Pattern of elution of ^{14}C-glucose from a silicic acid column.

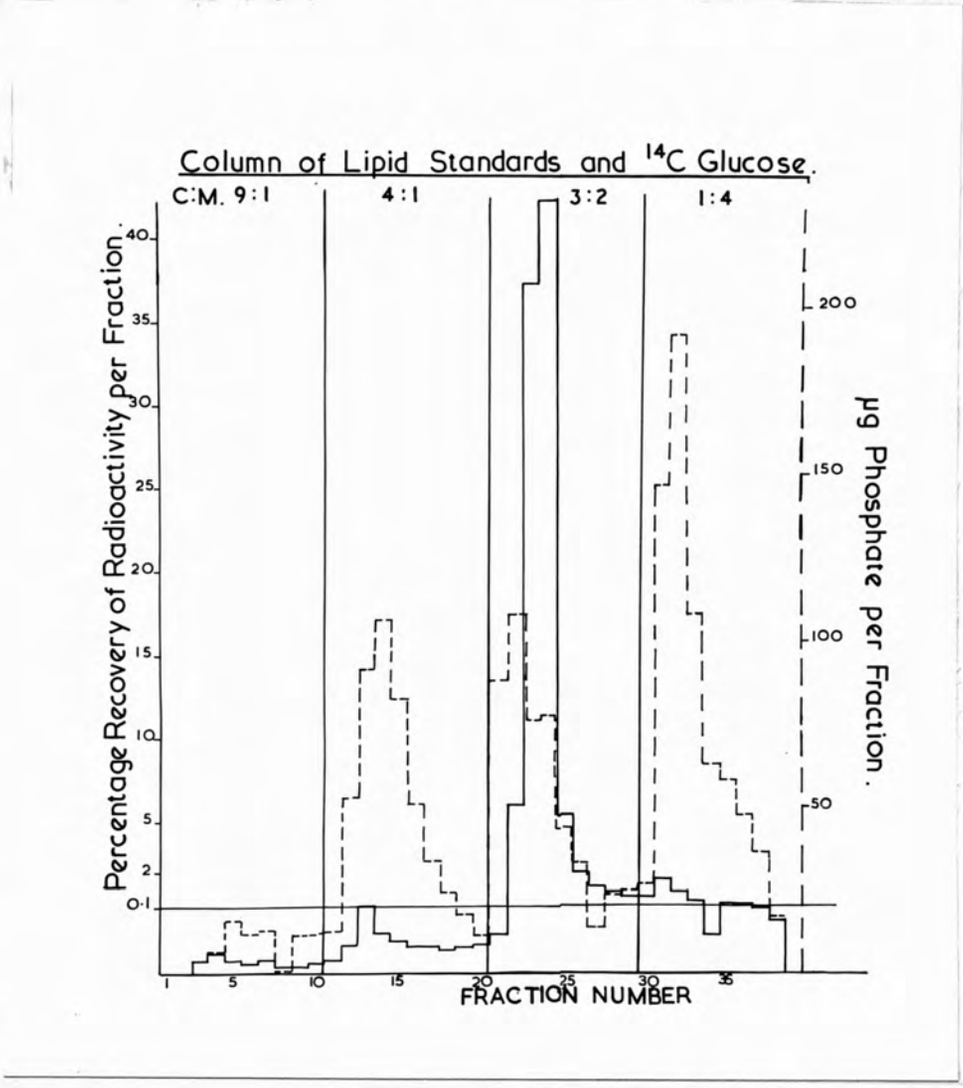


Fig. 10.1. (b) - Pattern of elution of ^{14}C-glucose (solid line) from silic acid column in presence of lipids. Elution pattern of phospholipids is indicated by the phosphate content of fractions (broken line).

Extractions of Preparations Treated with ^{14}C-Glucose.

Work on the fractionation of red cell lipids was continued with the aim of isolating a possible glucose complex. This possibility was encouraged by the findings of labelling from inhibitors amongst the lipid fractions. Since most of the inhibitors used appear to have been fairly nonspecific it was considered that the surest method of isolating the component involved would be to obtain it in a complex with glucose. This obviously presents many difficulties as the complex is very labile, but the problem was approached in several different ways as described below.

Chromatographic behaviour of free glucose.

In order to determine the distribution of free glucose in the systems used a column and thin layer plates were run using ^{14}C-glucose alone. From silicic acid columns the radioactivity was eluted mainly in fractions 23 and 24, (early chloroform:methanol 3:2) with a small degree of trailing through the remainder of this solvent and into the next chloroform:methanol 1:4. The radioactivity remained chiefly at the origin and did not move more than 0.5 cm from it when applied to thin layer plates which were developed in chloroform:methanol: water 80:30:3.

To find if the chromatographic behaviour of glucose was affected by lipids on the column a mixture of standard lipids and ^{14}C-glucose was separated on a silicic acid column. This also enabled the behaviour of lipids on the column to be checked. A mixture of

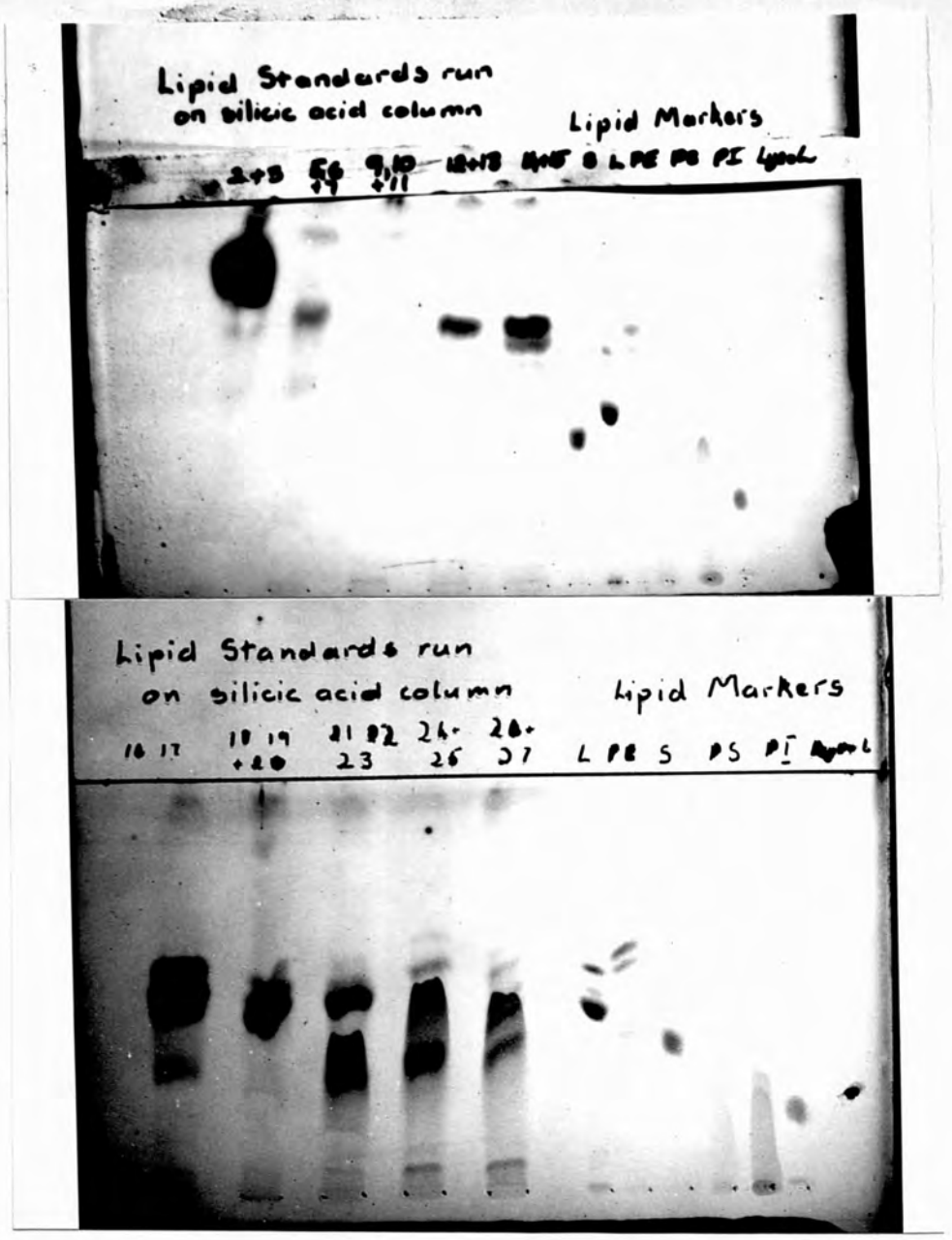
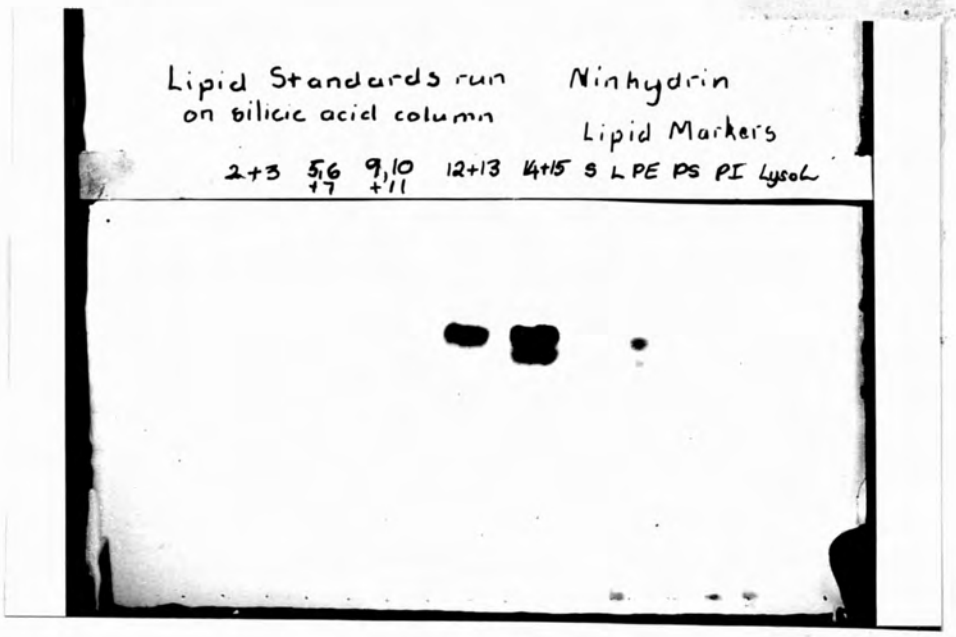


Fig. 10.2. (a).

T.L.C. of lipid standards recovered from a silicic acid column developed in the usual way - stained with iodine.

Fractions pooled as shown.

Lipid markers - L - lecithin; PE - phosphatidyl ethanolamine; S - sphingomyelin; PS - phosphatidyl serine; PI - phosphatidyl inositol, LL- lysolecithin.



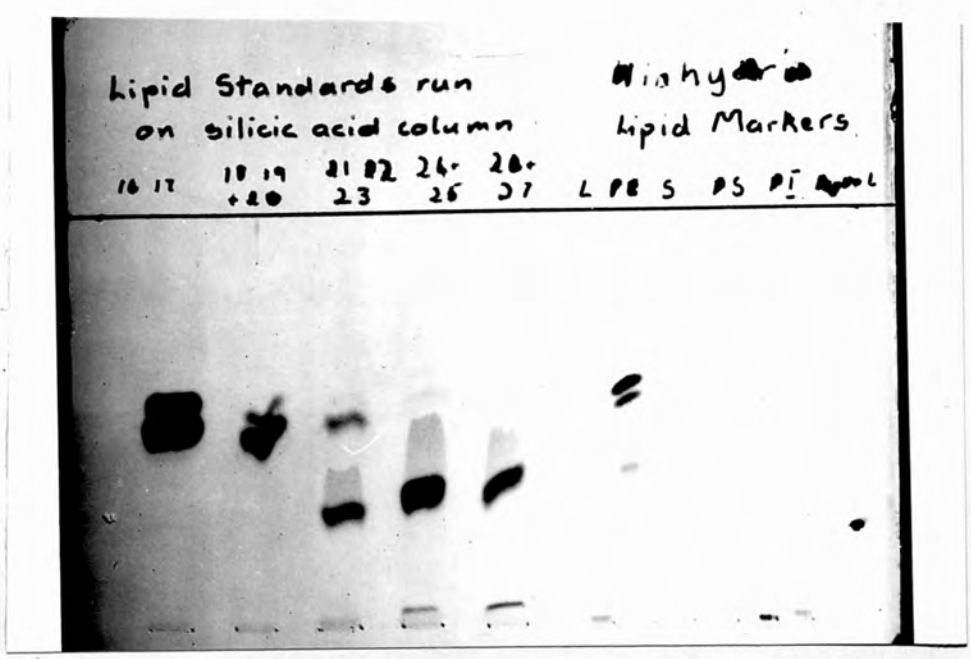
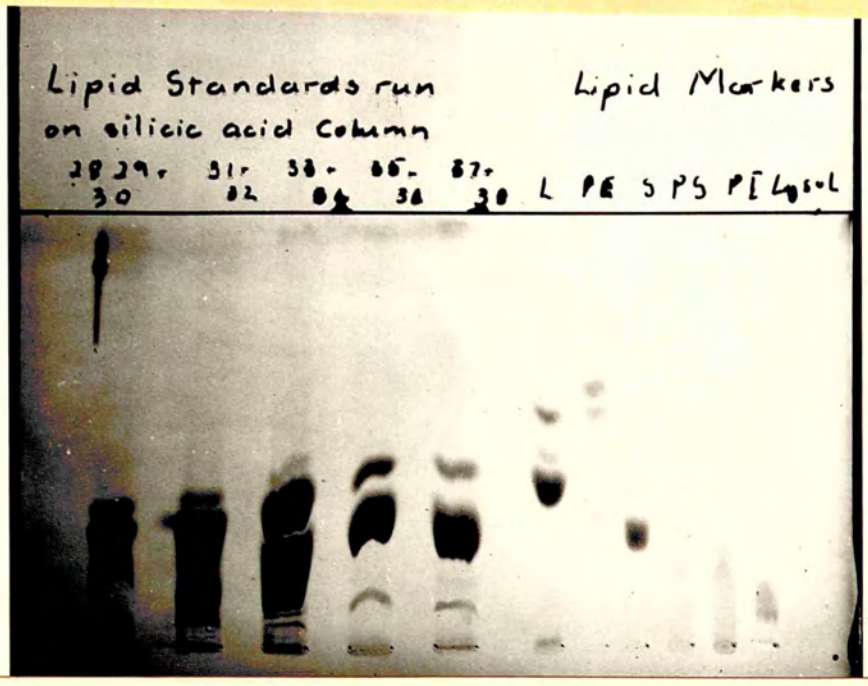


Fig. 10.2. (b) - Plates as shown in fig. 10.2. (a) but stained with ninhydrin.

(i)



(ii)

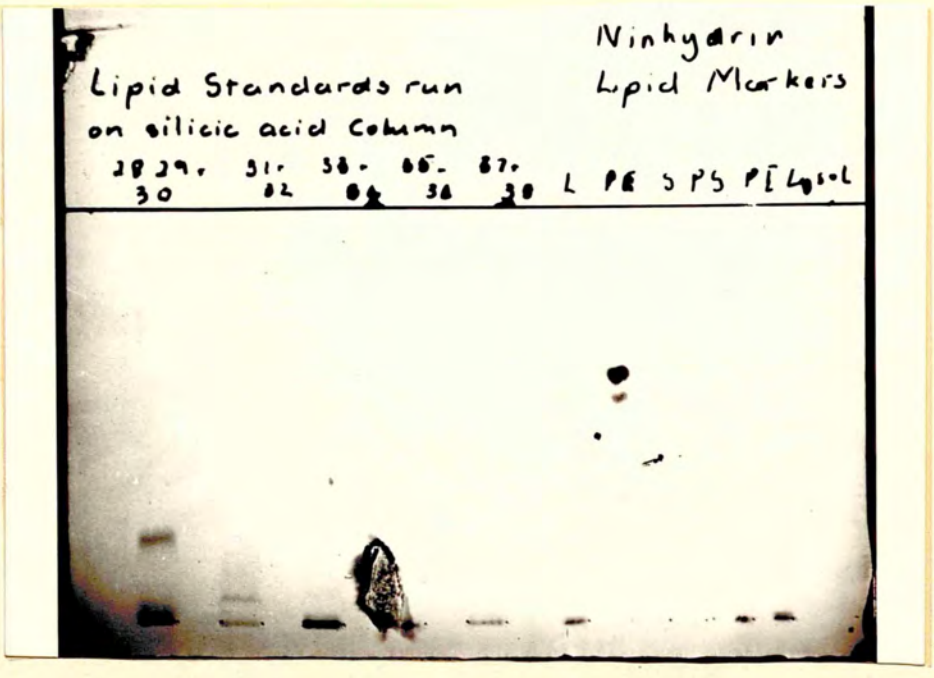


Fig. 10 2. (c) - T.L.C. of lipid standards recovered from a silicic acid column - developed in chloroform:methanol:water 80:30:3 stained with (i) Iodine and (ii) Ninhydrin. Fractions pooled as shown.

Lipid markers - see fig. 10.2. (a).

lipids was prepared as follows: 50 mg cholesterol, 15 mg lecithin, 10 mg phosphatidyl ethanolamine, 10 mg phosphatidyl serine, 10 mg sphingomyelin, 25 mg phosphatidyl inositol and 2.5 mg lysolecithin in 2 ml. chloroform:methanol 9:1. To this was added 12.5 microcuries ^{14}C-glucose in 1 ml. chloroform:methanol 9:1. The material was separated on a silicic acid column (10 g silicic acid) samples being taken for phosphate determination and counting. Virtually the same distribution of radioactivity was obtained as for glucose alone, but there was a very low percentage present in virtually all the tubes indicating that the lipids can carry along minute quantities of glucose. It seems unlikely that glucose actually complexed with any of the lipids.

The separation obtained on the column was as expected. Cholesterol was recovered chiefly in fractions 2 and 3. There were also some fast running lipids present in these early fractions which were not found in the lipid markers and probably represented breakdown products formed during separation and storage. The majority of phosphatidyl ethanolamine was recovered in fractions 12-17. Phosphatidyl inositol and phosphatidyl serine appeared in fractions 18-27. Lecithin was eluted in fractions 22-27, lysolecithin in fractions 33 and 34 and sphingomyelin in fractions 33-38.

The recovery of radioactivity and lipid was close to 100%

<u>The effect of DNFB on the uptake of ^{14}C-glucose.</u>

The purpose of these experiments which were run in conjunction

with DNFB uptake and isolation and experiments described earlier was to compare the uptake of glucose in the presence and absence of DNFB. It was hoped that this would help to clarify the action of DNFB and indicate whether it acted by superimposing itself upon the glucose at the transport site or by displacing it. If it were in fact superimposing itself on the glucose complex, the sugar would be fixed as irreversibly as the DNFB and since the complex formed would probably remain stable during extraction procedures, this could be important to further work.

Time expired erythrocytes were used which had been washed three times with phosphate buffer and tested for glucose exits.

In the first experiment with glucose alone, 4 ml. ^{14}C-glucose (5 micro curies/ml.; 176,000 counts/min), were added to ice cold cells, shaken for 20 seconds for extraction.

In the second experiment using DNFB, 50 ml. red cells were equilibrated with 76 mM non-radioactive glucose at 37°C for 30 minutes and 5 ml. ^{14}C-glucose solution for 20 seconds and sufficient alcoholic DNFB added to give a 10 mM solution. The cells were shaken with DNFB for 60 seconds before being extracted with 500 ml. cold n-butanol. It was hoped that the metabolic incorporation of ^{14}C-glucose would again be minimized and that the high concentration of DNFB would bring a degree of inhibition in this short time. Before extraction a portion of cells was removed to measure the time course of glucose exits. Inhibition was found to be of the

order of 10%.

The extracts were filtered and evaporated to near dryness under nitrogen. The chloroform soluble lipids were obtained by adding chloroform to the slightly wet extract, mixing gently and spinning. The precipitate was extracted with chloroform:methanol 1:1 and spun, after which there was still a small quantity of solid material remaining which was suspended in carbon tetrachloride and which dried to a white powder. The recovery of radioactivity and lipids from red cells incubated with ^{14}C-glucose in the presence and absence of DNFB is shown below:-

Solvent used to disolve material.	DNFB Absent.		DNFB Present.	
	Dry Wt.	% Recovery of RA.	Dry Wt.	% Recovery of RA.
a. Chloroform	215 mg	9.3	140 mg	0.8
b. Chloroform:methanol 1:1	7 mg	1.0	135 mg	3.55
c. Insoluble material	7 mg	0.3	90 mg	4.4
Total recovered	230 mg	10.6	365 mg	7.5

The percentage recoveries in the two experiments were quite high and although the recovery of radioactivity from each experiment was not greatly different, the relative percentage of labelling in each fraction was quite different. A quantity of radioactivity in the insoluble material in experiment 2 probably represented free sugar, as there was a large quantity initially present in this experiment.

Table 10.1.

Uptake of ^{14}C Glucose by Human Red Cells

in Presence and Absence of DNFB.

Separation of butanol extracted lipids on silicic acid Hyflosupercel columns, 8, 7 and 9.

Chloroform soluble lipids - without DNFB.

Eluting Solvent.	Fractions Eluted.	RA cts/min.	% cts.	µgP.	Dry Wt. (mg)
9:1	1 - 5 (I)	355	0.11	20.3	6.4
9:1	6 - 10 (II)	1,315	0.42	63.0	34.2
11:2	11 - 14 (III)	446	0.12	38.0	1.0
11:2	15 - 19 (IV)	1,675	0.5	105.75	3.8
4:1	21 - 26 (V)	2,187	0.69	190.5	5.9
4:1	27 - 30 (VI)	30,740	9.73	502.5	10.0
3:2	31 - 35 (VII)	59,400	18.77	396.0	16.7
3:2	36 - 42 (VIII)	202,465	63.2	288	26.0
1.4	42 - 44 (IX)	13,115	4.17	106.5	16.6
1.4	45 - 49 (X)	3,590	1.12	826	36.7

Eluting Solvent.	Fractions Eluted.	RA cts/min.	% cts.	ugP.	Dry Wt. (mg)
	Chloroform soluble lipids - with DNFB.				
9:1	1 - 4 (I)	1,100	0.8	58.3	42.3
9:1	5 - 8 (II)	945	1.9	222.5	14.4
9:1 & 11:2	9 - 12 (III)	235	0.4	152.5	5.5
11:2	13 - 16 (IV)	1,035	2.1	65	4.0
11:2 & 4:1	17 - 20 (V)	900	2.2	112.6	1.5
4:1	21 - 28 (VI)	3,610	5.4	168.8	5.1
3:2	21 - 31 (VII)	7,366	15.2	112.75	4.4
3:2	32 - 37 (VIII)	33,574	68.8	317.5	16.4
1:4	38 - 41 (IX)	560	1.1	330.0	11.6
1:4	62 - 46 (X)	301	0.6	317.55	10.5
	Chloroform:methanol soluble lipids - with DNFB.				
4:1	1 - 6 (I)	305	0.5	43.75	1.0
4:1	7 - 11 (II)	345	0.55	18.75	1.0
3:2	12 - 15 (III)	380	0.9	27.5	4.2
3:2	16 - 20 (IV)	24,900	40.9	47.5	17.7
1:4	21 - 24 (V)	32,450	53.2	50.4	25.0
1:4	25 - 29 (VI)	620	2.8	37.5	5.8
1:9	30 - 35 (VII)	385	0.65	28.75	0.4
1:9	36 - 40 (VIII)	170	0.3	18.75	0.4

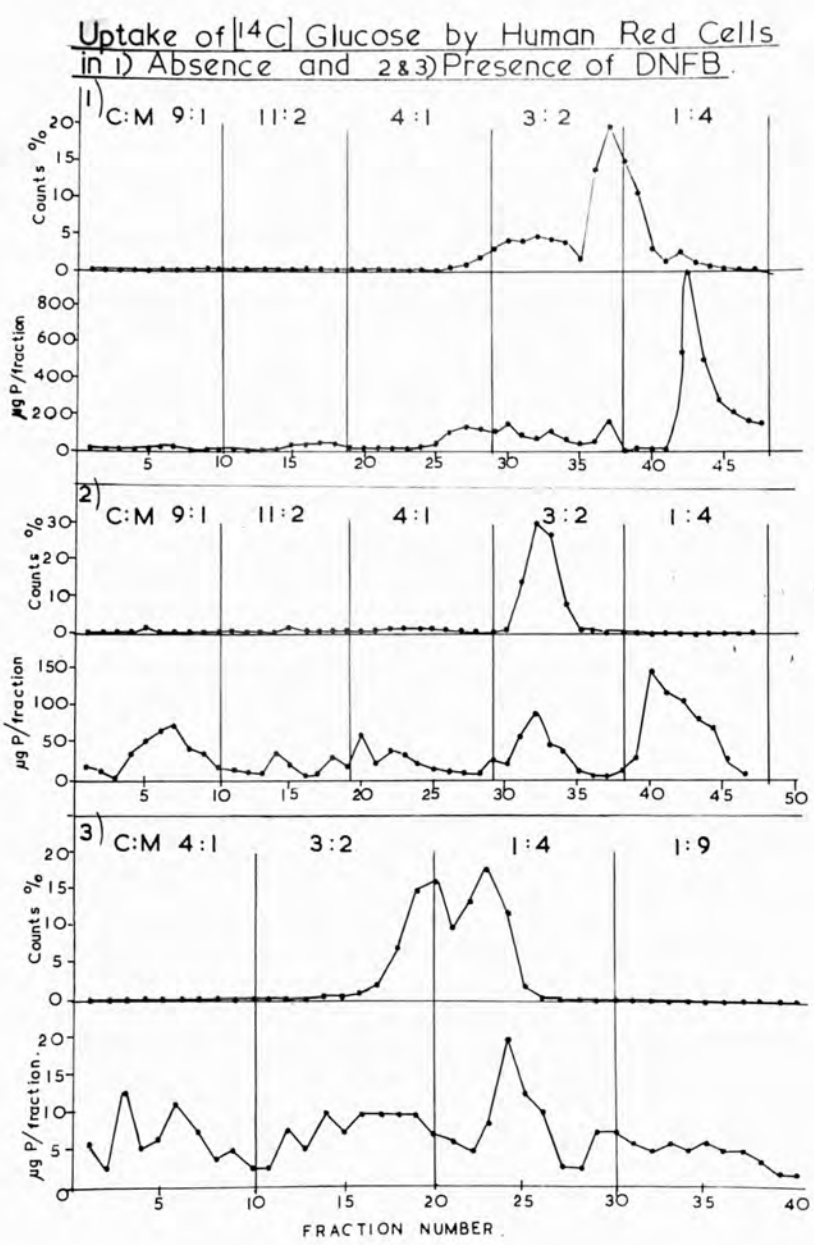


Fig. 10.3.

Three column chromatograms of phospholipids extracted with butanol from human red cells incubated with ^{14}C-glucose and subsequently in 2 and 3 with DNFB. Eluting solvents as usual.

1. Column 8 - 210 mg lipids (chloroform soluble) separated on 20 g silicic acid and 10 g Hyflo-supercel.
2. Column 7 - 130 mg lipids (chloroform soluble) separated on 15 g silicic acid and 7.5 g Hyflo-supercel.
3. Column 9 - 7.5 mg lipids (soluble in chloroform:methanol 1:1) separated on 13.5 g silicic acid and 6.8 g Hyflo-supercel.

It is also possible that DNFB displaced some sugar. The increase in weight in fractions b and c of experiment 2 was probably due to a considerable extent to the presence of unlabelled glucose as there were 0.75g in the original incubation mixture. The extracts were analysed by distribution chromatography on silicic acid columns as may be seen in fig. 10.3. Table 10.1. shows the detailed results for each eluting solvent.

Chloroform:methanol 11:2 was used as the second solvent in (1) and (2) to separate any polyphosphoglycerides present from cephalin. Chloroform:methanol 1:9 was used as a final solvent in 3 to elute the more polar lipids.

There appears no reason for the extremely large recovery of phosphate in the chloroform extract of experiment 1.

1. Column 8 - The fractions were pooled as indicated in Table 10.1. and small quantities of the material run on thin layer plates with lipid standards. The radioactivity in sample VIII obtained from the large peak of late chloroform:methanol 3:2 remained near the origin indicating free glucose, but much of that in VI and VII obtained from the low wide peaks in late chloroform:methanol 4:1 and early 3:2 separated into several spots. The majority of the material from VI, VII, VIII and IX were then run in wide bands on thin layer plates and eluted as shown overleaf.

Portions of VIa, VIb, VIIa, and VIIb were retained for separation on plates. The remainder and material from fractions VIII and IX were hydrolysed with 5N.HCL. When the HCL had been

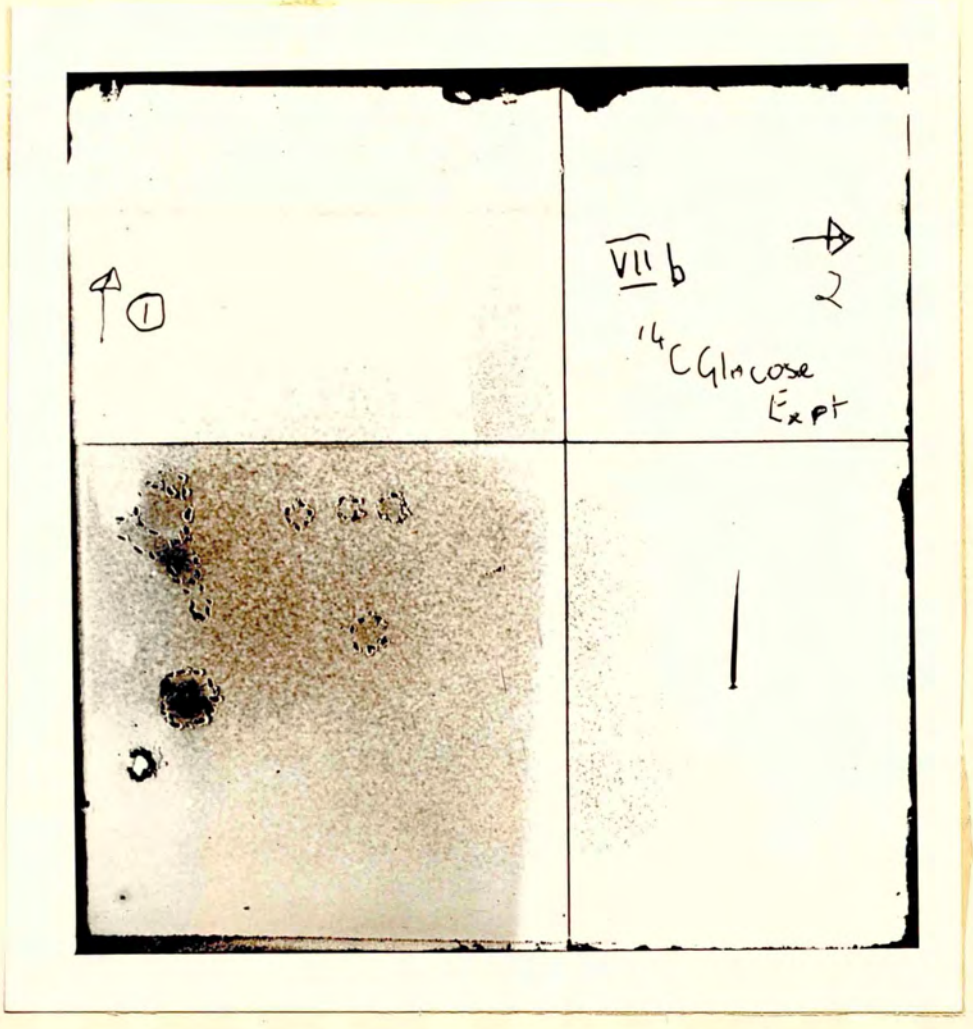


Fig. 10.4. - Two dimensional thin layer chromatograms of amino acids obtained on hydrolysis of sample VII b derived from thin layer plate. Subfraction of column no. 8 as described in the text.

Developing solvents.

1st. direction chloroform:methanol 17% NH_4 OH 2:2 lV/V.

2nd direction phenol:water 75:25 V/V stained with ninhydrin.

evaporated off the solution was tested for amino acid content.

Group	Rf of Elution.
VI a)	0.15 ..... 0.3
VI b)	0.5 ..... 0.75
VII a)	0.05 ..... 0.15
b)	0.35 ..... 0.55
VIII	0.05 ..... 0.3
IX	0.15 ..... 0.45

Strong positive ninhydrin reactions were given by VI and VII although VIII and IX gave a weaker reaction. The hydrolysates were run on a plate with five amino acids, alanine, aspartic acid, leucine, serine, and threonine. Several amino acids were present, some running with the markers. The samples where then run on two-way chromatograms, that of VIIb is shown in fig 10.4 Those amino acids present appeared to be leucine, valine, alanine, threonine, glycine and possibly aspartic acid and ~~glutathione~~ glutamine. No such clear separation of amino acids from VIII and IX could be obtained. The significance of these quantities of amino acids is not understood, but it would appear that a peptide chain may be present.

2. Column 7.- Fractions from the column were pooled as shown in the table and further separated by thin layer chromatography. The distribution of lipids was similar to that described in 1. The radioactivity in fractions I, II and II eluted by chloroform:methanol

9:1 and 11:2 was displaced slightly from the origin, associated with spots giving a slight colour with iodine and pale yellow due to the presence of DNP-derivatives. The cephalin was eluted over a wider range than in 1, appearing in 11:2, 4:1 and 3:2. Radioactivity from the large peak was similarly distributed to that in 1. In VII from the early part of the 3:2 peak the radioactivity appeared at Rf 0.3 and 0.5 with a smaller quantity at Rf 0.85. A quantity from the second half of the peak appeared with Rf 0.15 associated with lipids which were possibly inositides. A faster running fraction was also present. Separate plates were run of VII, VIII and II and the material eluted as shown below was subjected to hydrolysis with 5N HCL.

Group.	Rf of Elution.
VII a)	0.15 ..... 0.35
b)	0.35 ..... 0.55
VIII a)	0.03 ..... 0.27
b)	0.55 ..... 0.67
II	0.55 ..... 0.95

(II represents the fraction from which DNP amino acids were isolated in the earlier described experiment).

Separation of DNP amino acids from II in one direction seemed to indicate the presence of DNP ethanolamine, DNP leucine and possibly DNP alamine. They were further separated in two dimensions using first toluene: pyridine:ethylene chlorohydrin:0.8N ammonium hydroxide 100:30:60:60. The upper phase was used for development,

the lower for pre-treatment of the plate. The second solvent system was chloroform:tert. amyl alcohol:acetic acid 70;30:3. Five yellow spots were obtained of which 3 were identified as dinitrophenol, DNP ethanolamine and DNP leucine.

No free amino acids were found in samples II, VII and VIII after hydrolysis.

3. Column 9.- Fractions were pooled as shown in fig.10.3 and separated further on thin layer chromatograms. Peaks of radioactivity were eluted in the chloroform:methanol 3:2 and 1:4. Cephalin and lecithin were found in these fractions. On thin layer plates the radioactivity was found partly near the origin and partly associated with an earlier phospholipid spot at Rf 0.2. The lipids of chloroform:methanol 1:4 were essentially lysophosphatides. Fraction IV appeared to be the most interesting. The radioactive portion was eulted from the plate and hydrolysed but no amino acids were found.

These results show that glucose can become associated with phospholipids of human red cells and that this association occurs in the presence of DNFB. Glucose appeared to be associated with lipids eluted late from the columns. The presence of DNFB increased the uptake of ^{14}C-glucose to a level comparable with that in ice cold incubation mixtures. There is little evidence of DNFB in association with highly radioactive fractions but if it were present in quantities compatible with complexed ^{14}C-glucose then it would probably not not be detectable. DNFB did seem to affect the mobility of the

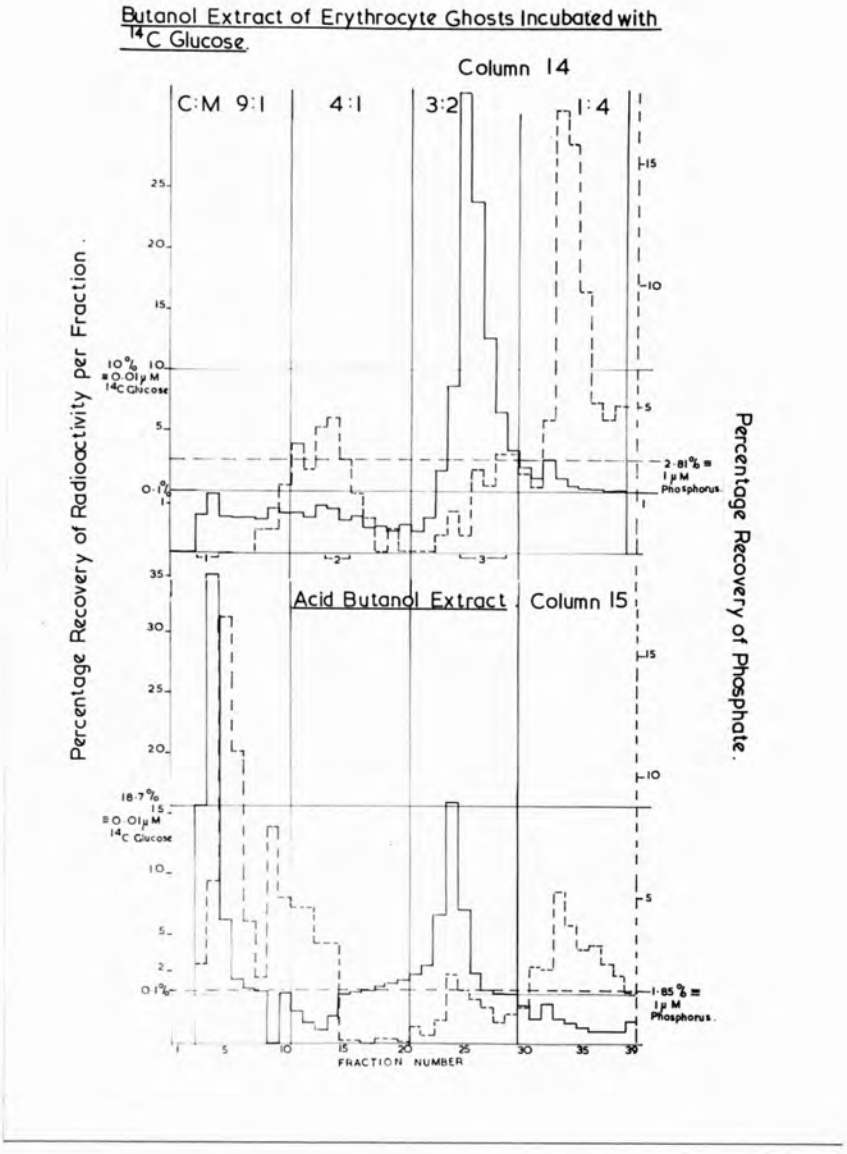


Fig. 10. 5. - Two chromatograms of phospholipids extracted with 1) butanol (column no, 14) and 2) acid butanol (column no. 15) from human erythrocyte ghosts incubated with ^{14}C-glucose. In each case 100 mg material was separated on 10 g silicic acid and 4 g Hyflo-supercel by elution with mixtures of chloroform: methanol.

Solid line represents elution pattern of radioactivity, and broken line that of lipid phosphate.

Note change of scale below 0.1% recovery of radioactivity and 1% for lipid phosphate.

cephalin fraction but the effect did not appear to be a simple acceleration one.

In these extractions separation and other procedures were hampered by the presence of haemoglobin. It was, therefore, decided to use erythrocyte ghosts in the following experiments. It was also possible that more of the reactive groups would be exposed.

Uptake of ^{14}C labelling by lipids obtained from erythrocyte ghosts.

These experiments were performed to see if it were possible to obtain labelling in lipids of erythrocyte ghosts after incubating with ^{14}C-glucose. Ten millilitres of glucose solution were prepared containing 0.07 mg glucose and total specific activity 50 µc. Sixty millilitres of ghosts were incubated with 9 ml. of this solution and extracted with 450 ml. butanol and 100 ml. acid butanol as described in Chapter 3.

Butanol extract - The total glucose added was 0.35 millimoles of which 0.198 millimoles were recovered in the butanol fraction. One hundred and fifty milligrams of material were obtained and dissolved in chloroform:methanol 9:1 in which all the material was soluble. There seemed to be no advantage in separating the lipids into two fractions according to solubility in chloroform. One hundred milligrams of the material were separated on 10 g silicic acid and eluted as shown in fig. 10.5. (note change in scale above 0.1% recovery of radio activity). Elution of phosphate

was essentially the same as that obtained with ghosts incubated with ^{14}C-NEM. From each solvent those fractions containing radioactivity were pooled.

Sample.	Fractions.	RA Counts/min.	Nanomoles.
1	3, 4	2,000	0.159
2	13, 14	1,900	0.150
3	25 - 28	1,207,800	96.0
4	32 - 34	59,000	4.8

Further examination on thin layer chromatograms showed that ^{14}C in sample 1 moved about the same amount as free glucose. Pooled fractions - 2 gave a double peak. When this material was run again after elution and dilution with free glucose (to determine the relative contribution of free glucose to the radioactivity) the radioactivity was found to occur earlier and in smaller quantities. In 3, run without glucose a large peak was found not displaced far from the origin which was not reduced when ^{12}C-glucose was added.

The material in 4 samples 25 - 28 was divided into two portions and 5 mg unlabelled glucose added to one. The material in each portion was then further separated on DEAE cellulose columns using the solvents already described. If any glucose lipid complex were obtained in chloroform:methanol 3:2 on the silicic acid column and masked by the free glucose then this procedure might have served to reveal it. The pattern of recovery of labelling was the same in both columns. The radioactivity was chiefly recovered with

chloroform:methanol 7:3 some trailing into the methanol. A very small proportion was found in chloroform:methanol 3:1 and chloroform: glacial acetic acid. The only difference between the two columns was that there was a small reduction in the radioactivity recovered from the second column - that run with material containing ^{12}C-glucose.

From the first DEAE column fractions 3, 4, 5 and 6 obtained with chloroform:methanol 9:1 and 7:3, fractions 13 and 14 (chloroform: methanol 7:3) and fractions 21, 22 and 23 (chloroform acetic acid 3:1 + 0.05 M NH_4Ac.) were pooled in those groups and re-run on thin layer plates in the presence and absence of free unlabelled glucose. The same procedure was carried out with fractions 5 and 6, 8, 9, and 10, 12, 13 and 14, 21, 22, 23 and 24 obtained with the same solvents from the second DEAE cellulose column.

The lipid components appeared to be mainly lecithin and a small quantity of phosphatidylethanolamine. The radioactivity was obtained in the form of free glucose. The wide distribution of radioactivity on the DEAE column was due to the poor separation of free glucose or to bound glucose being released on the column.

Acid butanol extract - The total recovery in the acid butanol extract was 0.064 millimoles glucose, 0.037 millimoles being obtained in fractions 2, 4, 5 and 6 and 0.18 millimoles in fractions 23-25, after separation of material obtained from the extract on a silicic acid column. The butanol acid extract was interesting

in that not only was free glucose present in chloroform:methanol 3:2 but labelling occurred in the early peak associated with a quantity of lipid phosphate. The fractions which constituted each of the two radioactive peaks were pooled and run on thin layer plates, the radioactive parts eluted and re-run. The material from fractions 23-25 appeared to be free glucose but that from the earlier fractions appeared in a position 5.5 cm from the origin. The material associated with radioactivity was eluted and hydrolysed with 6 N HCl. The hydrolysate gave a positive ninhydrin reaction but when chromatographed in two dimensions with chloroform:methanol:17% ammonia 2:2:1 and phenol:water 75:25, the amino acids could not be identified and did not appear to be associated with radioactivity. Thus it appears possible to obtain a small degree of ^{14}C-labelling (from glucose) associated with lipids extracted by butanol and a considerable quantity with those extracted by acid butanol

Uptake of ^{14}C-Glucose in the Presence of Phloretin.

The action of phloretin as a competitive inhibitor has already been described. This compound would be expected to prevent glucose from combining with the membrane component postulated to be involved. It was, therefore, considered that a reduction in labelling of any one fraction in the presence of phloretin could indicate the involvement of this component in membrane transport.

A suspension of ghosts (40 ml.) were shaken with 10 ml. of 1:1,000 phloretin solution for 3 minutes and then 10 ml. of ^{14}C-

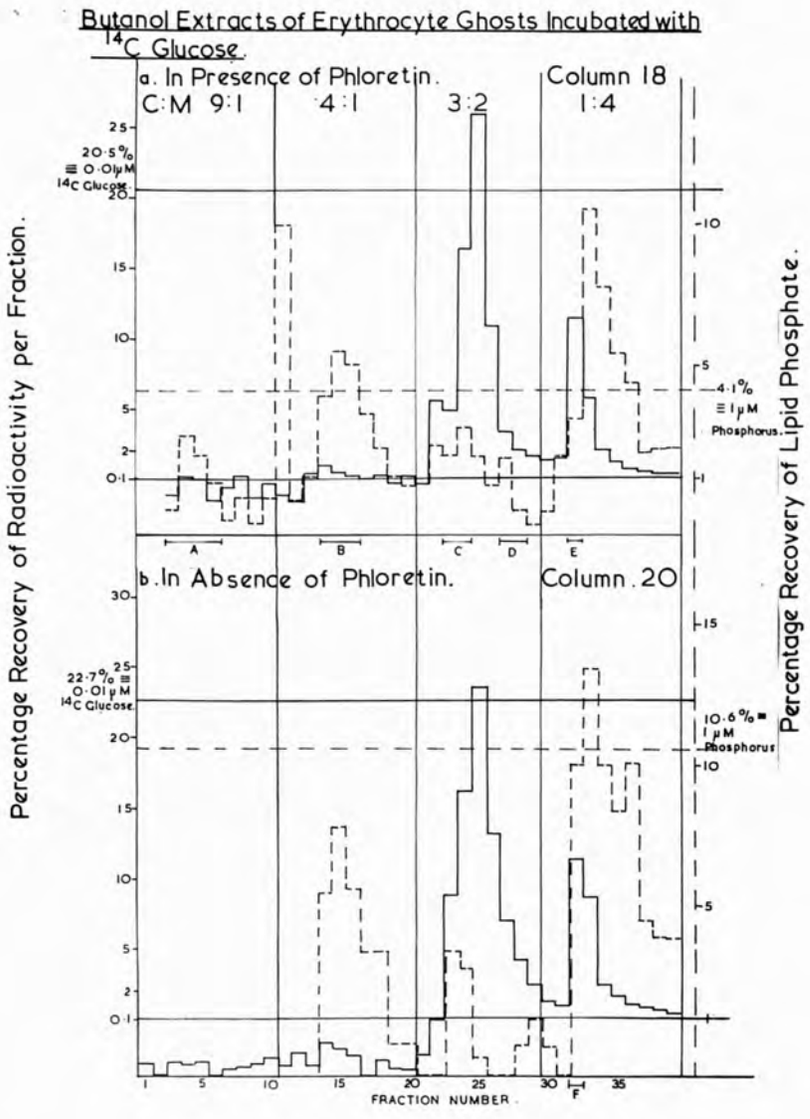


Fig. 10.6.

Column chromatograms of phospholipids extracted with butanol from human erythrocyte ghosts incubated with ^{14}C-glucose in presence (column no. 18) and absence of phloretin (column no. 20). Material eluted as usual. Solid line represents elution pattern of radioactivity and broken line that of phosphate.

Note change of scale below 0.1% on recovery of radioactivity and below 1% for phosphate recovery. Fractions were pooled for further separation on thin layer plates as indicated below ABCD etc.

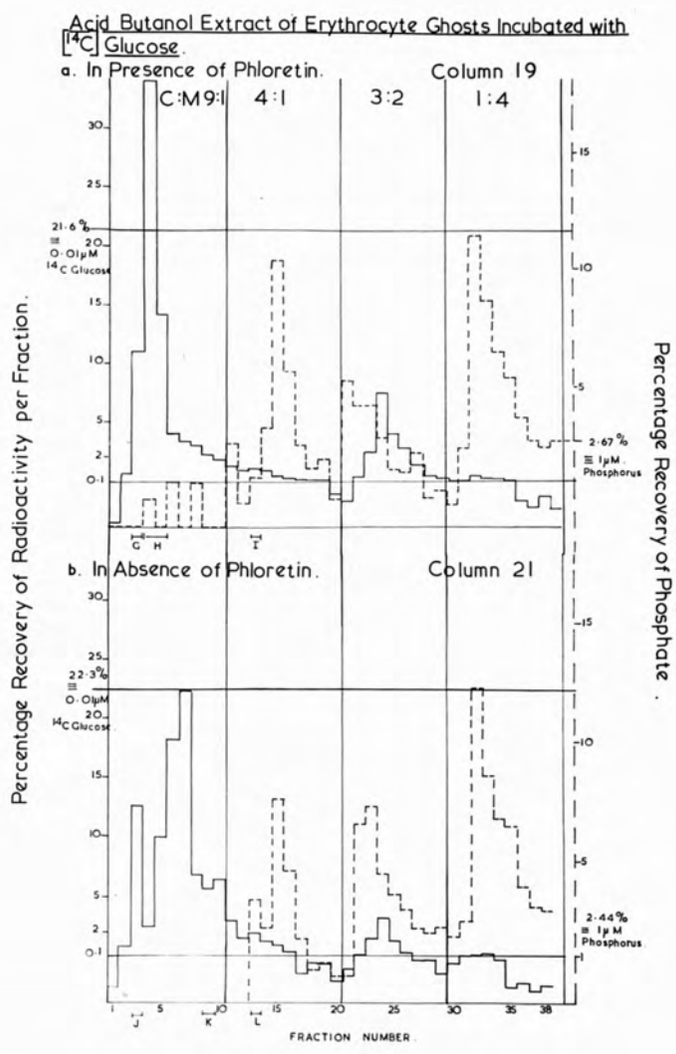


Fig 10.7. - Column chromatograms of phospholipids extracted with acid butanol from human erythrocyte ghosts incubated with ^{14}C-glucose in presence (column no. 19) and absence (column no. 21) of phloretin.

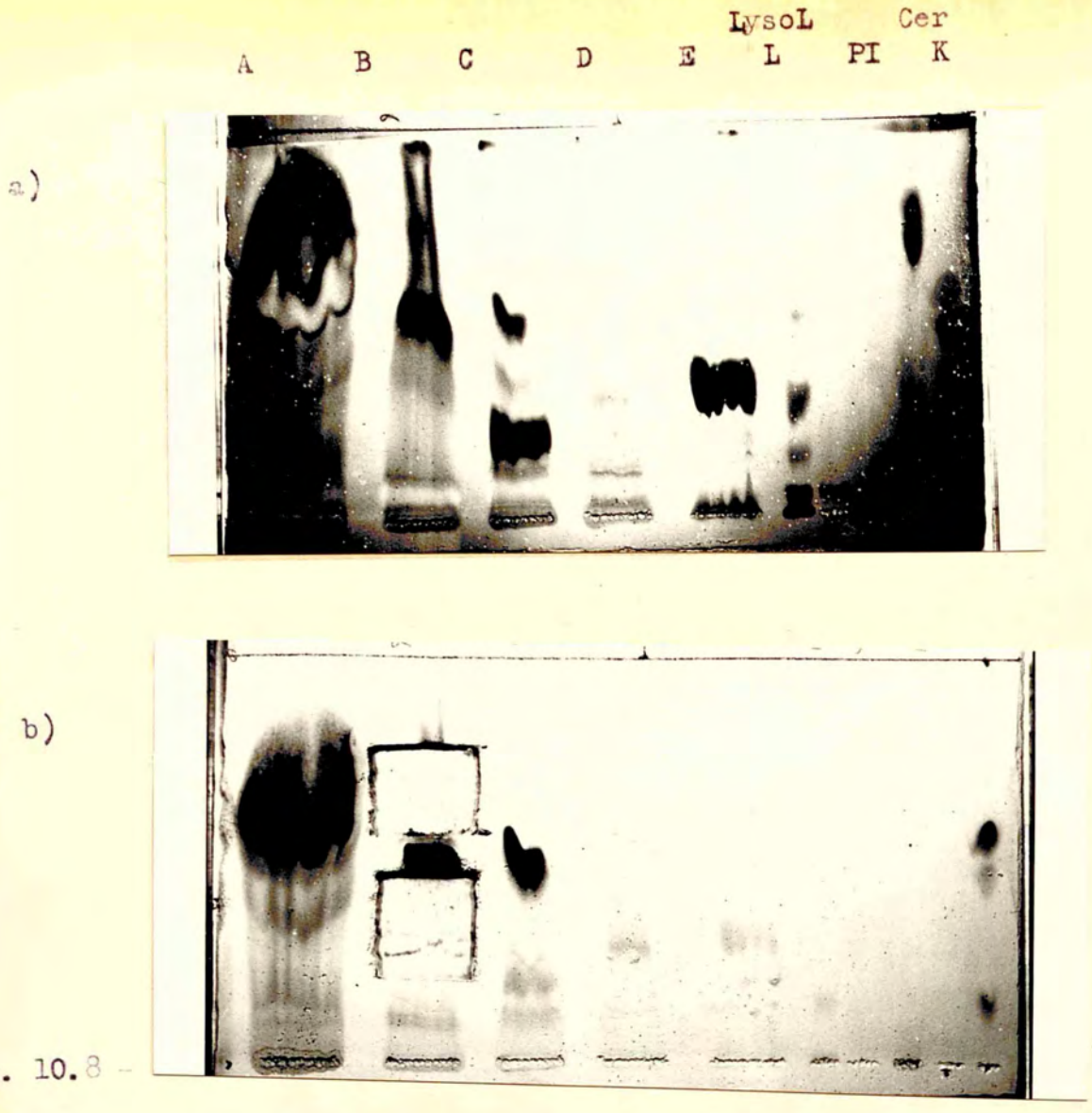


Fig. 10.8 -

Thin layer chromatogram of butanol extract of red cell ghosts which had been incubated with phloretin and then ^{14}C-glucose.

Plates developed as usual and stained with a) iodine; and b) ninhydrin.

Fractions pooled as shown in fig 10.6.

Lipid markers - L - lecithin; LysoL - lysolecithin; PI - phosphatidyl inositol; Cer - cerebroside; K - cephalin.

Note - portions of B removed for further analysis.

loose association.

Acid butanol extract - The radioactivity appears in the acid butanol fraction from phloretin treated cells with almost identical distribution to the control, but the high phosphate associated with the early peak is absent. These results are difficult to explain in terms of the considered mode of action and could be better explained if it did not affect glucose complexing directly.

None of the radioactivity from the acid butanol ran as free glucose on the plates, that in G and J running to the solvent front and that in H and K running with Rf 0.6. The peak of radioactivity in H was found to have an Rf of 0.42 and 0.6. Thus we seem to be dealing with the same material in the corresponding fractions in the two columns. The material eluted in column nos 18-21 seemed of some interest and tests carried out by Dr. D.A. Nixon, St. Mary's Medical School, London, revealed the presence of a small quantity of inositol.

These experiments while seeming to indicate that glucose is able to complex with some substance to become lipid soluble show little evidence of the action of phloretin.

Results of experiments extracting ghosts pre-treated with ^{14}C-glucose may be summarised as follows:-

	Extract.	Glucose.	Phosphate.	Phosphate/glucose.
First Experiment	Butanol (column 14)	0.1 µM	35.6 µM	356
	Acid butanol (column 15)	0.64 µM	84.5 µM	1,320
Phloretin	Butanol (column 19)	0.049 µM	20 µM	400
	Acid butanol (column 21)	0.046 µM	37.5 µM	820
Control	Butanol (column 18)	0.044 µM	9.45 µM	215
	Acid butanol (column 20)	0.045 µM	41.0 µM	910

glucose solution (total activity 40uc) for one minute. They were extracted overnight with 250 ml. butanol and the residue extracted with acid butanol. A control was prepared by shaking a further 40 ml. ghosts with 10 ml. saline for three minutes adding 10 ml. ^{14}C - glucose and extracting with butanol and acid butanol. Column chromatography of the four extracts in choloroform:methanol 9:1 gave separation as shown in figs. 10.6 and 10.7.

Butanol extract - Of interest is the fact that radioactivity appears in solvent 1:4 in the butanol extracts. This is not seen in other controls. In this later peak in the control, the elution of glucose appears to be ahead of the phosphate, raising the question whether this could represent change in elution of lipids associated with glucose.

Thin layer chromatograms shown in fig. 10.8 indicated that samples from column 18 (lipid extract treated with phloretin) contained radioactivty mainly in the form of free glucose. In samples A, C and D radioactivity appeared with an Rf 0 - 0.05, that in B appeared at the origin and with Rf. 0.31. and while in E it streaked from 0.1 - 0.19. The material in B was eluted as shown and re-run when a second spot appeared with Rf 0.65. Of the radioactivity in F (column 20), as well as that appearing at the origin, there was a small quantity which appeared with an Rf of 0.24. Labelling obtained in chloroform:methanol 1:4 could represent material carried along indiscriminately with lecithin or glucose held in

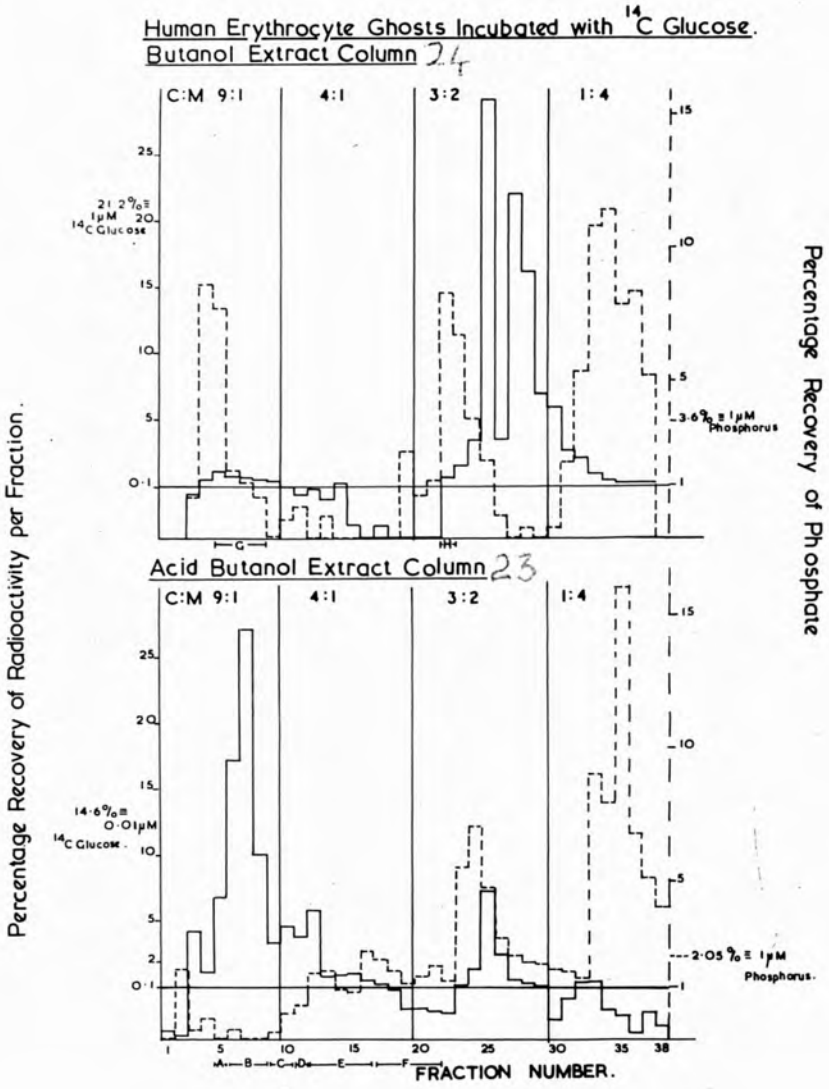


Fig. 10.9.

Column chromatograms of phospholipids extracted with 1) butanol (column no. 24) and 2) acid butanol (column no. 23) from human erythrocyte ghosts incubated with ^{14}C-glucose - eluted as usual .

Solid line represents elution pattern of radioactivity and broken line that of phosphate.

Note change of scale below o.1% recovery of radioactivity and 1% for lipid phosphate.

ABCD etc. indicate fractions pooled for further investigation.

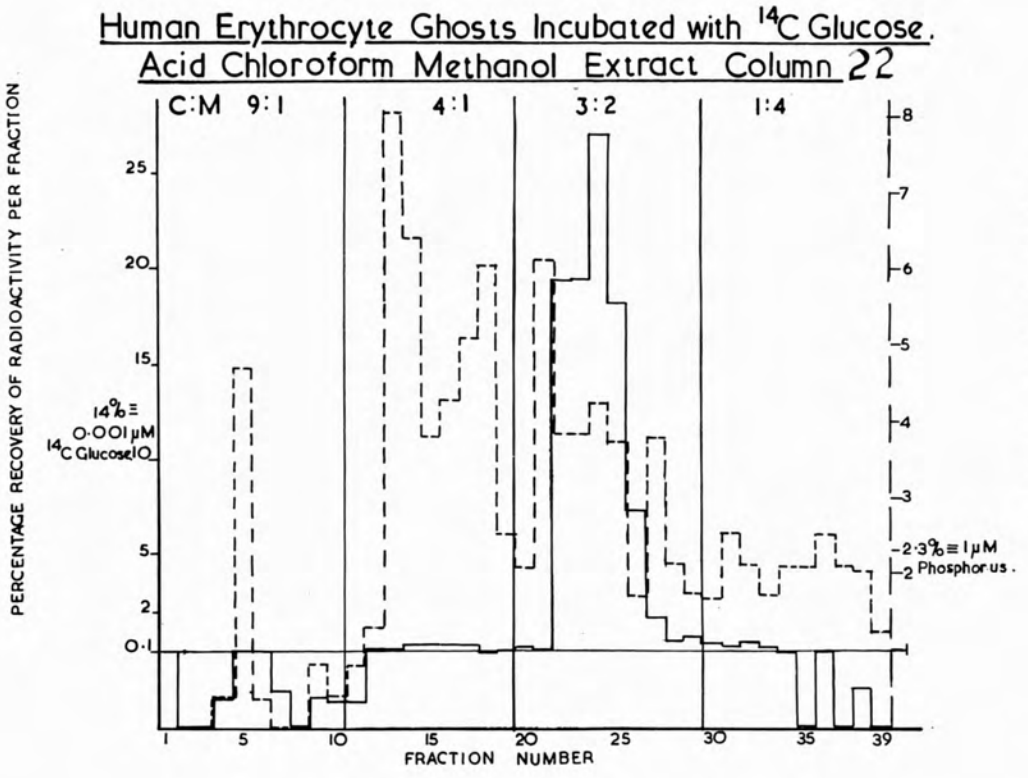


Fig. 10.10. - Column chromatograms of phospholipids extracted with chloroform (column no. 22) and separated as for fig. 10.9.

If glucose were associated with a phospholipid a constant phosphate/glucose ratio would be expected. Those for butanol and acid butanol extracts are of the same order. Too much emphasis cannot be placed on these results as only a small quantity of the lipid and glucose are complexed.

Extractions using Butanol and Acid Butanol and other Solvents in ^{14}C Experiments.

In view of the inconclusive results obtained with phloretin a further examination of extracts was made.

Butanol and acid butanol extracts were made of 65 ml. ghosts incubated with ^{14}C-glucose and also extracts with chloroform:methanol 1:1 and acid chloroform:methanol. After extraction with chloroform two phases separated out, a lower chloroform and an upper water methanol layer.

Recovery of ^{14}C-labelling in the various fractions was as follows:-

Solvent.	Volume obtained (ml).	Counts.	mMoles ^{14}C-glucose.
Butanol	400	1,764,000	0.217
Acid butanol	100	1,100,000	0.135
Water-methanol	117	2,161,000	0.267
Chloroform	255	517,000	0.064
Acid chloroform methanol	150	423,000	0.052

Separation of the components of the mixture was carried out on silicic acid columns as shown in figs. 10.9 and 10.10. which also shows samples used for thin layer chromatography. The pattern obtained with butanol and acid butanol was similar to earlier experiments.

It is interesting to note that with acid chloroform:methanol there is a similar distribution of radioactivity to that obtained with butanol extractions, the majority of radioactivity appearing in the fractions where free glucose appears. There is no large early peak, but a small quantity of radioactivity is eluted in all fractions which is not found when glucose alone is run.

The methanol water fraction contained only free glucose, presumably any bound glucose would be removed by hydrolysis.

The fatty acids of Samples B (containing 0.35 mg glucose) E (containing 4.5 lipid phosphorus) F and L were hydrolysed and methylated using the following method. The material was dried and refluxed for 90 minutes with 10 ml. methanol:benzene:sulphuric acid 150:75:7:5. To this were added 10 ml. distilled water and 10 ml. ether. The ether and water fractions were allowed to separate and both retained. All the radio activity appeared in the water fraction. The methyl esters of the fatty acids were separated by Drs. C. Hitchcock and B.W. Nichols (Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford) using gas liquid chromatography. Unfortunately the material was not present in large or pure enough quantities for positive identification, but the fatty acids of F and L were similar, but present in different ratios. B was different again.

Samples A, C and K were run on thin layer plates using chloroform: methanol:water 80:30:3 as a developer. The radioactive areas Rf 0.4 - 0.5 and 0.55 - 0.65 and 0.5 - 0.6 respectively were eluted and the plate stained. The radioactivity appeared to be associated with

lipids and in sample K the ninhydrin reaction was especially pronounced. The material was obtained rather early from the column to be phosphatidyl ethanolamine, although the Rf value was similar to that obtained with ethanolamine. The material eluted was dried and taken up in ether. Five millilitres of ether solution were then shaken with 5 ml. water. The ^{14}C-labelling was recovered entirely in the water phase. Both water and ether fractions were taken to dryness and the material from the ether extractions of K and A was dissolved in chloroform: methanol 4:1. Material from C was divided into two portions, one part was taken up in chloroform:methanol 4:1 and the other in 4 vols chloroform and 1 vol methanol containing 93 mg% DNFB to determine whether the material obtained would react with DFNB. The ether soluble material was rechromatographed on thin layer plates. Very little lipid was recovered. There was lipid staining material which appeared with Rf 0.55 in K (the cephalin standard appeared as two spots Rf 0.55 - 0.65) and there was some ninhydrin positive material in A 7 cm from the origin. The DNFB ran chiefly as the free material.

The water extracts were taken to dryness and run on boric acid plates as described in Chapter 3 with glucose standards (10µl, 20µl, and 30µl of 0.23% solution of glucose). The radioactivity travelled to a distance of 3 cm from the origin whereas free glucose travelled 2.5 cm - 3 cm. It is possible that this could represent differences in Rf due to irregularities of the silica film, but it seemed that the glucose was combining fairly irreversibly with some group to give a

complex, which while soluble in lipid solvents possesses greater solubility in aqueous solvents. Glucosamine-glucose-1-phosphate and glucose-6-phosphate were also run on these plates, but did not correspond with radioactive spots. Uptake of labelling was unlikely to represent incorporation as there are few enzymes in the ghost and the experiments were initially carried out at 0°C. In these experiments it was possible to find labelling in a fraction where free glucose did not appear. The glucose seemed to have reacted in some way with material iosolated from the membrane. When material bearing ^{14}C-labelling was taken up in ether and shaken with water, the labelling appeared in the water but did not behave quite like free glucose on thin layer chromatography.

It was still not clear which quantity of labelling, if any, was of significane in glucose transport. Therefore, the following series of experiments were performed.

Effect of DNFB and ^{12}C-glucose on the uptake of ^{14}C-labelling.

DNFB could possibly be expected to reduce the uptake of labelling by any fraction involved in glucose transfer across the membrane and an excess of unlabelled glucose would almost certainly be expected to do so. Therefore, a series of experiments were set up as follows:-

1) 5.0 ml. of glucose (from a stock containing 5 µc in 25 ml.) were added to 40 ml. loosely packed ghosts and shaken for one minute and extracted with 300 ml. butanol at 4°C overnight.

Table 10.2.

Effect of DNFB and ^{12}C-glucose in the uptake of ^{14}C-labelling.

Experiment	% recovery in butanol extract of radioactivity added.	% recovery in acid butanol extract of radioactivity added.
1 - Control	37.3	23.6
2 - Cells + 0.94 mg ^{12}C-glucose	44.0	20.9
3 - Cells + 94 mg ^{12}C-glucose	35.3	31.1
4 - Cells incubated with DNFB	38.0	23.5

Uptake of ^{14}C-labelling by human and sheep erythrocyte ghosts.

Experiment.	% recovery in butanol extract of radioactivity added.	% recovery in acid butanol extract of radioactivity added.
1 - Human control	31.2	17.4
2 - Human cells + 0.94 mg ^{12}C-glucose	33.9	21.5
3 - Human cells + 94 mg ^{12}C-glucose	38.5	23.0
4 - Sheep ghosts	23.5	15.8

2) 0.05 ml. ^{12}C-glucose (18.9 mg/ml.) 4.95 ml. saline and 5.0 ml. ^{14}C-glucose were added to 40 ml. packed ghosts, were shaken together for one minute and then extracted with 300 ml. butanol at 4°C overnight.

3) 5.0 ml. ^{12}C-glucose and 5.0 ml. ^{14}C-glucose were added to 40 ml. packed ghosts, shaken for one minute and extracted with 300 ml. butanol at 4°C overnight.

4) 50 ml. ghosts were treated with 5 ml. DNFB solution, containing 107 mg in 1 ml. absolute alcohol and 4 ml. saline, for 15 minutes at 21°C. The cells were then chilled and shaken with 50 ml. ^{14}C-glucose solution for one minute and extracted with 300 ml. butanol at 4°C overnight.

The butanol from each extraction was decanted and the residue extracted with acid butanol. The recoveries of ^{14}C-labelling are shown in Table 10.2. The recovery was the same in all 4 experiments - about 64%. Silicic acid columns were run, the pattern of elution being identical with that in earlier experiments.

The recovery of radioactivity in the acid butanol peak in experiment 1 was 147,326 cpm or 7.366 nanomoles glucose, in experiment 2 it was 97,700 cpm so that 6.3 nanomoles were present and in experiment 3, 127,090 cpm equivalent to 6.3 nanomoles. The peak recovery with DNFB was 115,560 cpm i.e. 5.75 nanomoles glucose, which represents a 27% decrease on that obtained in experiment 1. The results of experiment 3, however, could indicate that the peak material is of no great importance as it was not reduced despite the excess of ^{12}C-glucose present.

The components of fraction 6 from the first 3 columns were further

separated by thin layer chromatography. The only material present in any quantity was lipid material positive to ninhydrin with Rf 0.5 - 0.6., similar to that of the cephalin marker. The radioactive material from fraction 6, experiment 3, was eluted, dried down, taken up in 7 ml. ether and shaken with 5 ml. water. All the radioactivity was obtained in the water phase. The ether soluble material was re-run and one lipid spot, positive to ninhydrin was obtained near the solvent front. The water soluble material was run on boric acid plates with 20 µg and 40 µg glucose, the spots were stained with aniline phthalate and scanned. Radioactivity was found 6 cm from the origin, well ahead of of free glucose. Similar results were obtained with fraction 6 from experiments 1 and 2, and fractions 8, 9, and 10 experiment (1). Fraction 6, experiment 1 was re-run in chloroform:metharol:water 80:30:3 and the radioactivity still ran to the same position. All fractions contained a reddish pigment thought to be haematin, so after running on plates the material from 8, 9 and 10 was eluted and its absorption spectrum in the ultra violet region examined. Maxima were obtained at 240, 402, 489 and 600 mµ - those of haematin are at about 542 mu and 579 mu.

Half of the material from 8, 9, and 10 was used for ether water partition experiments and the remainder hydrolysed and tested for free phosphate, but none was found to be present. If radioactivity in these fractions were indeed associated with phospholipid, about 0.09 µmoles of inorganic phosphorus should have been present. Haemoglobin derivatives were present in these fractions, therefore it was possible that

glucose might have reacted to form sorbitol or gluconic acid. Separation of these subtstances on thin layer plates seemed to rule out this possibility. Radioactivity in fraction 5, column 27, experiment 1, was eluted from a thin layer plate which had been run in chloroform: methanol:water 80:30:3 and taken up in ether, which was divided into three portions and shaken with ether distilled water. N/1,000 HCL (pH 4.5) or N/10 NH_4OH (pH 9) and the aqueous material re-run on thin layer plates. All the radioactive material was recovered in the aqueous layer and when run on boric acid plates, travelled with an Rf of 0.4 - 0.5, so the material did not seem to be affected by pH

Methyl esters of fatty acids from fractions 5, 7, 13 and 15,16 and 17 pooled (expt. 1), were examined using gas chromatography. They were first examined for lipids, being chromatographed in chloroform:methanol:acetic acid:water 85:15:10:4 and the plates scanned. 5 gave a single peak of radioactivity of about Rf 0.5. 7 gave two peaks, a major one with the same Rf and a minor one having Rf slightly higher. The major spot co-chromatographed with phosphalidyl ethanolamine, but when sprayed with concentrated suplhuric acid and charred did not exhibit the same colour characteristics. The esters were prepared in the following way:- half of each sample was taken to dryness and refluxed in 10 ml. 10% methanolic NaOH for 90 minutes, concentrated, acidifed and extracted with ether. The concentrated ethereal solution was treated with fresh diazomethane for 10 minutes and the volume reduced to approximately 100ul.

The fatty acid content was as follows:-

Chain length		14	16	18:0	18:1	18:n	18:2
Percentage in Fraction	Fraction 5	8	49	13		24	
	Fraction 7	8	52	12		24	
	Fractions 14, 15 and 16	1	31	19	21		4
	Fraction 31	2	33	17	22		14

In a further attempt to see if the complex involved in glucose transport could be found, the following series of experiments were performed. Sheep red cells were used as glucose transport is much slower than in human red cells and experiments with human red cells using large quantities of unlabelled glucose were repeated.

Uptake of ^{14}C-Labelling by Human and Sheep Erythrocyte Ghosts.

1.) Control - 50 ml. human erythrocyte ghosts were incubated with 5 ml. ice cold saline and 5 ml ^{14}C glucose for 60 seconds and then extracted with 300 ml. butanol at 4°C overnight.

2.) - 60 ml. of human erythrocyte ghosts were incubated with 0.05 ml. ice cold glucose (1.89%), 4.95 ml. saline and then 5 ml. ^{14}C-glucose for 60 seconds and then extracted with 300 ml. butanol overnight.

3.) - 50 ml. human erythrocyte ghosts were incubated with 5 ml. glucose 1.89% and then 5 ml. ^{14}C glucose for 60 seconds and extracted with 300 ml. butanol at 4°C overnight.

4.) 25 ml. of sheep erythrocyte ghosts were incubated with 5 ml. ice cold saline 5 ml. ice cold saline 5 ml. ^{14}C-glucose for 60 seconds and then extracted with 150 ml. butanol at 4°C overnight.

The percentage of radioactivity recovered is shown in Table 10.2.(page 205) It increases with increased glucose probably because a smaller proportion of labelling was lost as free glucose. The quantity of radioactivity recovered in sheep cells was down in both fractions as compared with the human red cells, but it must be remembered that only half quantities of cells were used.

Acid Butanol Extracts- The material recovered in acid butanol was separated on silicic acid columns, the results being identical with those in earlier experiments with acid butanol.

Taking the half saturation constant for glucose as 0.3 mM at the temperature used, the fraction of carriers saturated in experiment 1 is 0.0135, in experiment 2, 0.3 and in experiment 3, 0.98. There was, however, 18 times as much glucose in the peak of 3 as compared with that in 2. Thus experiment 3 substantially excludes the material eluted early as a complex of biological significance unless it could have been shown to be inhomogenous on thin layer chromatograms or unless the product was favourably made in acid butanol.

It is interesting to note that more labelling appeared in the early than in the late peak in experiment 4 where sheep red cell ghosts were used than experiments using human red cell ghosts.

Fractions 7 from experiments 3 and 4 and fraction 24 of experiment 4 were chromatographed. The radioactivity in fraction 24 was obtained in the position of free glucose, just displaced from the origin, that in fraction 7, experiment 4, had an Rf of 0.5 and that in 7 experiment 3 an Rf of 0.55. Radioactivity was confined to one main peak.

The possibility that the material obtained in acid butanol fractions was produced during extraction had, therefore, to be seriously entertained.

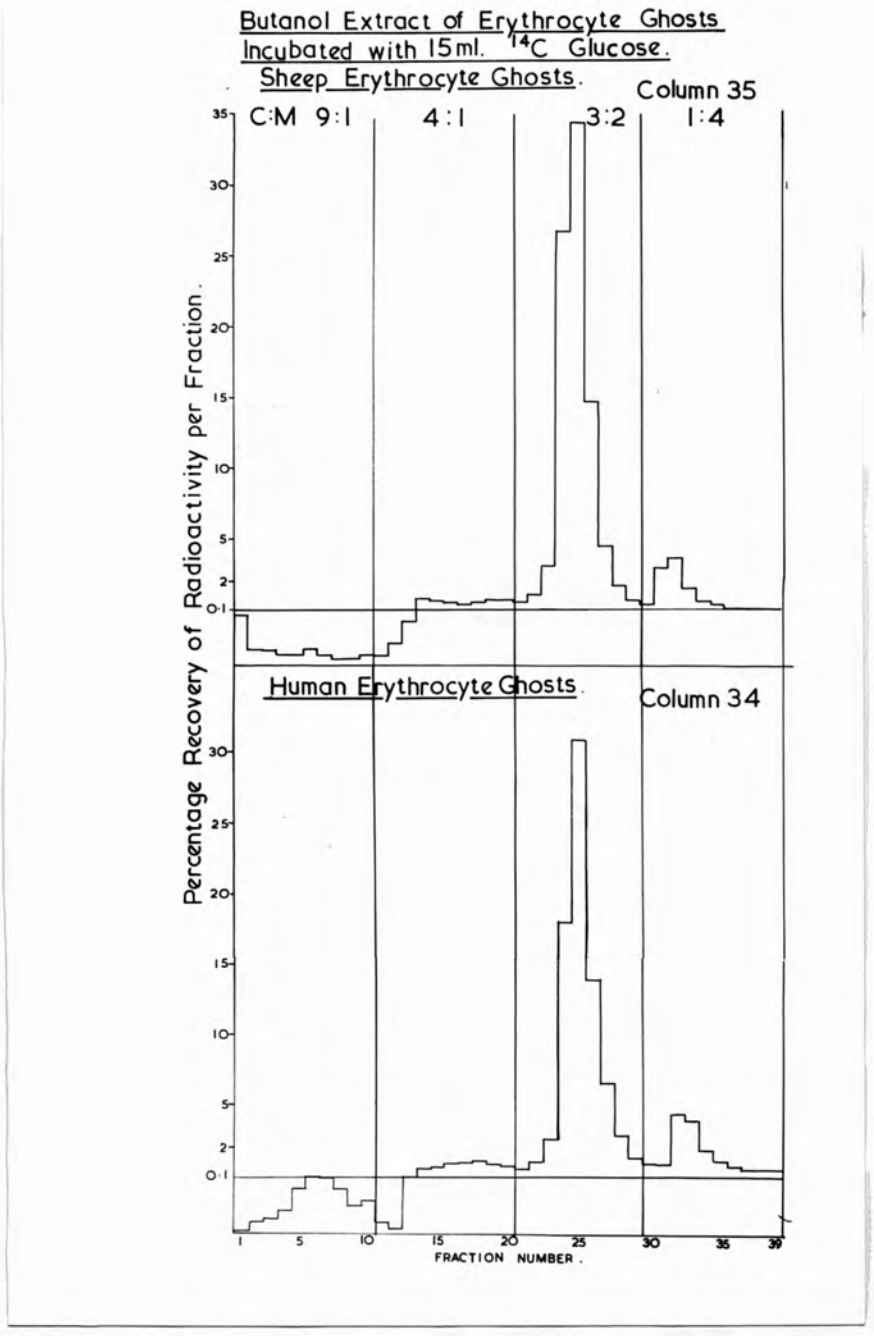


Fig. 10.11 - Elution pattern of ^{14}C-glucose recovered in butanol extract after incubation with 1) Sheep erythrocyte ghosts (column 35) and 2) Human erythrocyte ghosts (column 34).

Methyl alchol was first shown to react with glucose, in the presence of undissociated HCl to give methyl glucose by Fischer in 1893. The treatment afforded to the extract was not as vigorous as that used in the preparation of methyl glycoside but it was still possible that the butyl derivative was formed. Therefore, 25 ml. acid butanol were incubated with 1 ml. ^{14}C-glucose solution (27 nanomoles) at 45°C for three hours and then evaporated to dryness. When the material was run on a plate a radioactive spot with Rf 0.6 was found. Thus it appears that the glucose complex obtained in acid butanol extracts is in part at least a product of the extraction procedure.

Butanol Extracts - The process of column chromatography was applied to the butanol soluble material from experiment 1 as shown in fig 10.11. The majority of radioactivity was eluted in chloroform:methanol 3:2 where free glucose appears but significant quantities were also found in fractions eluted with chloroform:methanol 4:1 and 1:4. Fractions 15 and 16 (pooled) and 18 and 32 were further separated by thin layer chromatography which seemed to indicate that the radioactivity in 18 and 32 was due to free glucose. A double peak was obtained in fractions 15 and 16 with Rf 0.15 and 0.25. Radioactive containing material was eluted from the plates and re-run. When the plates were scanned, the distribution of radioactivity seemed to indicate that free glucose was present in all the fractions. It is possible that if glucose were complexed with lipid it would become dissociated during the running of the plate.

A further test of whether any of the labelling recovered were complexed would be to partition the labelling between a lipid solvent

Table 10. 3.

Distribution between ether and dimethyl formamide of radioactivity associated with lipid fractions.

Sample.	Total Counts.	Counts Initially in Ether.	% Counts in Ether.	Final Ether/ Formamide Ratio.
Column 34 Fractions 17-19	12,500	8,450	67.5	0.22
Column 34 Fraction 25	144,000	12,465	8.7	0.0009
Column 34 Fractions 33, 34	21,120	17,965	84.5	0.04
Column 35 Fractions 14, 15 & 16	4,500	4,350	97	0.09
Column 35 Fraction 25	16,080	550	3.1	0.0005
Column 35 Fractions 31, 32	7,651	230	3.02	0.007

and one in which glucose was preferentially soluble but which would not, as water, tend to dissociate the complex. Preliminary experiments seemed to indicate that dimethyl formamide would be suitable for this purpose. Free glucose passes almost entirely into the dimethyl formamide layer when shaken with petroleum ether and dimethyl formamide.

Samples from the column were dried down, taken up in 5 ml . petroleum ether and filtered into a small conical tube when 0.2 ml. of the solution was taken for counting. To the residue were added 2 ml. dimethyl formamide. The solution was filtered through the same funnel to extract any residue and 0.2 ml. taken for counts. The two extracts were shaken together, the volumes measured and further samples of 0.2 ml. taken for counting. The results are shown in Table 10.3. It would seem that some radioactivity is bound to lipid in fractions 17 and 19 since the ether/dimethyl formamide ratio is considerably larger (0.22), than in the other fractions, it being 0.009 in fractions 25, where radioactivity exists almost entirely as free glucose, and 0.04 in fractions 33 and 34. Of course the possibility that some lipid sugar complex passes into the dimethyl formamide cannot be entirely excluded.

The butanol extract in experiment 4 (sheep erythrocyte ghosts) was separated on a silicic acid column. Distribution of radioactivity was similar to that in experiment 1, suggesting that the material in 14 and 15 experiment 1, is not of great interest. However, it could be that the mechanism for glucose transfer is present in the sheep erythrocyte, but not functional, so that complexes could be formed.

Ether:dimethyl formamide partition experiments were carried out with fractions 14, 15 and 16 (pooled) 25 and 31 and 32 (pooled) from

experiment 4, the results being shown in Table 10.3. The ether:formamide ratio is low in all fractions; that in 14, 15 and 16 being higher than in the other two. Thus it appears that very little, if any of the glucose was firmly complexed with lipid material.

Before passing on to further extraction methods, it would be useful to summarise the main points obtained for butanol extractions. With the butanol extract, peak activity seemed always to be found in fraction 25 where free glucose is found. Of the acid butanol columns only 1 in 12 had the peak at 25, in 11 it was 24 or earlier . This discrepancy is a little surprising and would not be expected of free glucose. In the five columns - 14, 18, 20, 24 and 34 - all of butanol extracts, radioactivity was found in fraction 14, a higher percentage being found in some. The recoveries were as follows:-

Column 14	Fractions 13 and 14	1,900	counts per min.
18	" 13 and 14	7,000	" " "
20	" 14 and 15	440	" " "
24	" 13 and 14	840	" " "
34	" 13 and 14	3,100	" " "

The further examinations made of these fractions seemed to indicate that the ^{14}C glucose obtained here is present in some form of complex.

Activity in fraction 32 eluted after the main radioactive peak is consistently present, but from the column run with free glucose it appears that this could be partly due to trailing.

A small and varying, but persistent recovery of radioactivity was obtained in fractions 2 - 8, peaks being in fractions 4, 8, 5, 5 and 6 in the columns mentioned above. This could be butyl glycoside, but

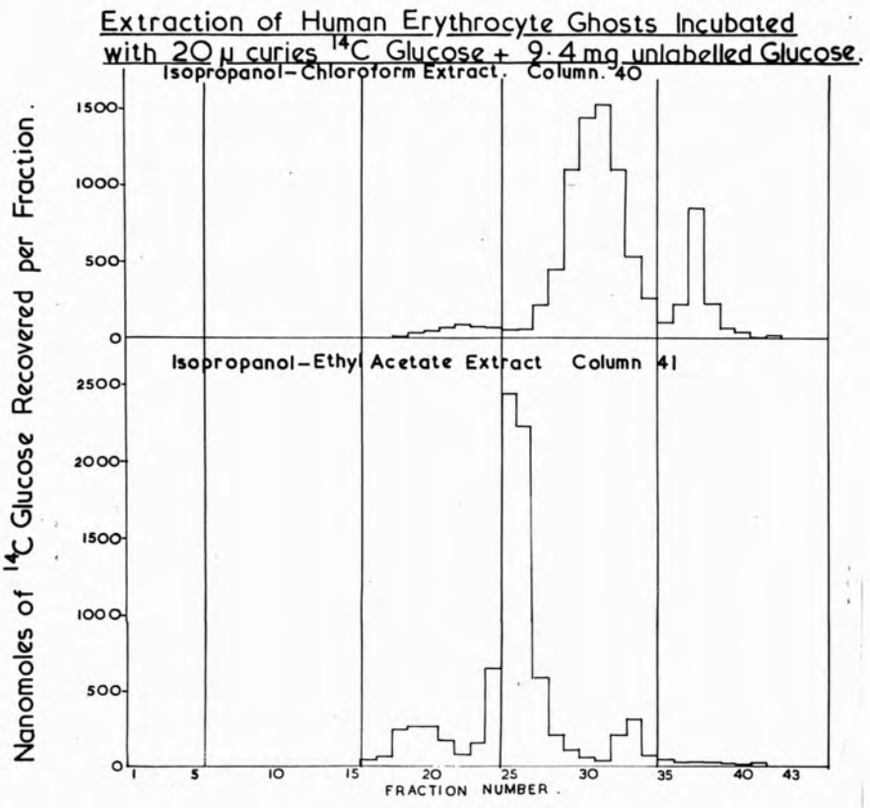


Fig. 10. 12. - Elution pattern of ^{14}C-glucose recovered in 1) isopropanol:chloroform extract (column 40). 2) isopropanol:ethyl acetate extract (column 41) after incubation with human erythrocyte ghosts. The eluting solvents were chloroform, chloroform: methanol 9:1, 4:1, 3:2 and 1:4.

could also represent a complex.

Extractions using Isopropanol.

Contrary to earlier experiments which seemed to suggest that butanol would be a useful solvent, those described above indicate that no satisfactory separation of a glucose lipid complex could be obtained using this solvent.

Rose and Oklander (1965) recommended isopropanol:chloroform as a suitable solvent system for the extraction of erythrocytes. Therefore further extraction of ghosts incubated with ^{14}C-glucose was performed using isopropanol:chloroform and isopropanol; ethyl acetate.

Two quantities of 80 ml. of human erythrocyte ghosts were treated with 9.45 mg ^{12}C-glucose and then 29 µg ^{14}C-glucose, total activity 20 µc. To each was added 495 ml. ice cold isopropanol After one hour 315 ml. chloroform was added to one flask and 315 ml ethyl acetate to the other. Extraction was allowed to continue for a further period of one hour and then the solid material was filtered off and stored under acetone. Further investigation of this material showed it to be of little interest. Approximately 300 mg lipid was obtained in each isopropanol extraction of which half was separated on silicic acid columns (fig 10.12). The distribution of radioactivity was as follows:-

Experiment A.- Isopropanol:Chloroform		Experiment B - Isopropanol Ethyl: Acetate	
Fraction	µmoles glucose	Fraction	µmoles glucose
21 - 25	0.322	18 - 21	0.925
28 - 33	6.158	23 - 28	6.246
36 - 38	1.296	32 - 34	0.577

Of these the early and late peaks represent 1.618 µmoles in

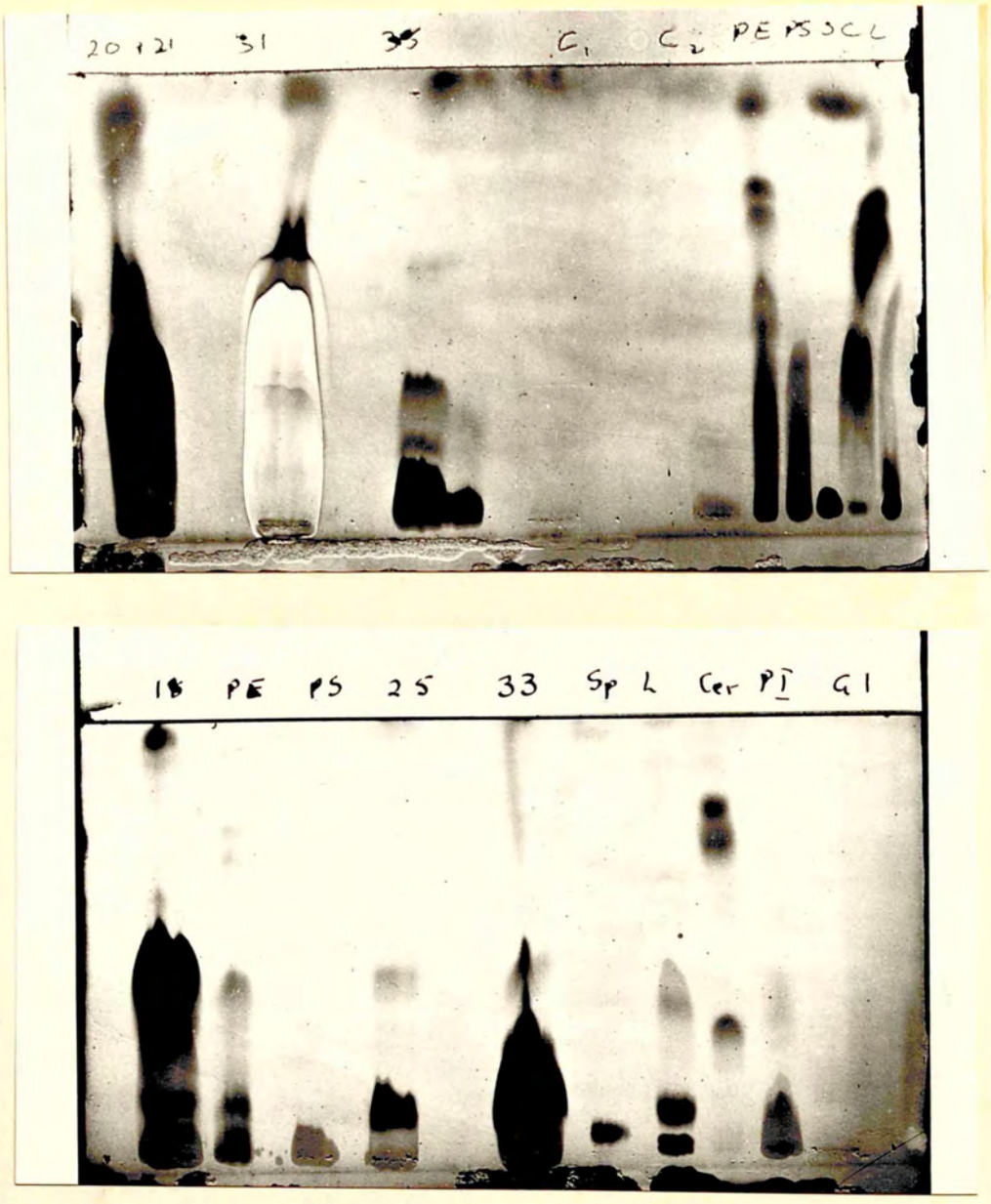
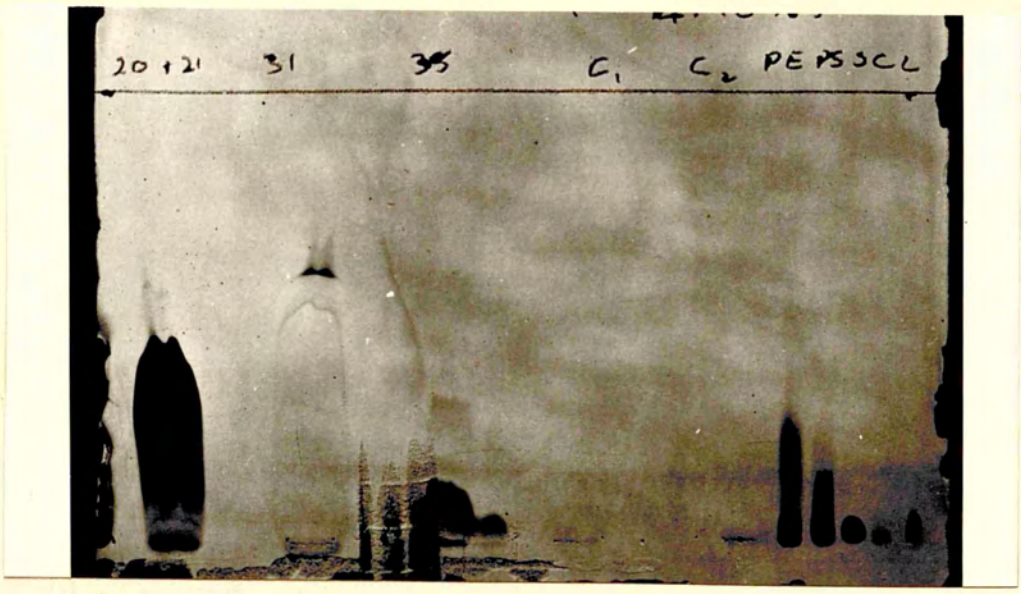


Fig. 10.13a- Thin layer chromatograms of lipids extracted with a) isopropanol:chloroform and b) isopropanol ethyl acetate after incubation with ^{14}C-glucose.

Plates developed in usual way and stained with iodine Fractions run as shown. G - free glucose. Lipid markers: PE phosphatidyl ethanolamine. PS phosphatidyl serine, Sp & S sphingomyelin, C & Cer cerebroside, L lecithin, PI phosphatidyl inositol.



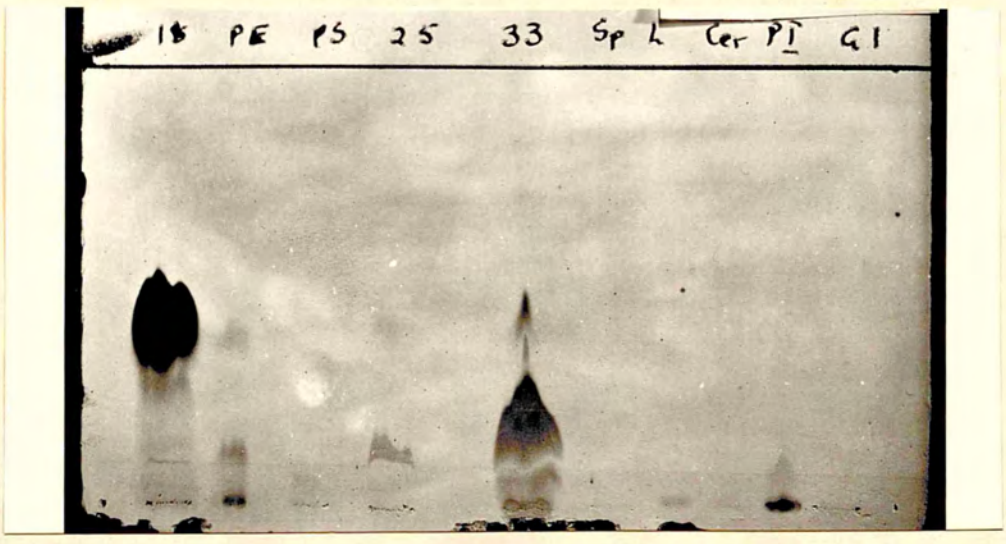


Fig. 10.13.b - As for fig. 10.13.a but stained with ninhydrin.

experiment A and 1.502 in experiment B - very similar figures and suggestive of the fact that the two peaks contain essentially the same material but in a slightly different form and hence running differently. Radioactive elution appears to be displaced five fractions to the right i.e. towards the eluent of greater polarity in the isopropanol:ethyl acetate extract. This, however, is not seen in the phosphate.

Samples from the three main peaks of each experiment were run on thin layer plates. They were 20 and 21 (pooled) 31 and 38 from the isopropanol:chloroform extract (experiment A) and 18, 25 and 33 from the isopropanol:ethyl acetate extraction (experiment B). The results are shown in fig. 10.13. In both, the early peak seems to be associated with the fraction containing mainly phosphatidyl ethanolamine, and the latter peak with slow running ninhydrin positive lipids. Only a small quantity of lipid was obtained from the peak with very high activity. That from experiment A seemed to contain chiefly phosphatidyl ethanolamine, and that in experiment B chiefly lecithin (C, C_1, and C_2 represents quantities of ^{14}C-glucose evaporated down with the two solvents to see if the possibility of reaction with the solvents could be eliminated.) The radioactive material from experiment B appeared to run chiefly as free glucose, but in fractions 20 and 21 ran slightly faster than free glucose and a double peak was obtained with fractions 31 and 38 at 0.5 and 2.5 cm and 0.5 and 2 cm respectively from the origin.

Ether:dimethyl formamide distribution was as follows:-

Experiment.	Fraction Number.	Counts.	Ether Soluble. Counts.	Nanamoles in Ether.	% Soluble in Ether.	Ether/ D. M. F.
1.	A	22-24	12,050	12,300	240	100	0.075
2.	A	29	27.765	5,875	117	17.5	0.002
3.	A	37	40.145	37,900	758	94	0.01
4.	B	19-20	23,040	18,350	367	78	0.063
5.	B	25	35,000	3,825	76	8.6	0.027
6.	B	32.34	33,760	22,450	449	67	0.010

It appears that if a complex exists in groups 3 and 6 it is quite easily broken down. The higher ether:dimethyl formamide ratio in 1 and 4 suggests the presence of a more stable complex. It is possible that the same results would be obtained with water but a less polar solvent is required.

It appears that material of some interest can be obtained with isopropanol:chloroform. To determine whether material obtained is of physiological interest or simply formed during extraction and concentration, the next series of experiments were carried out.

Uptake of Labelling by Ghosts extracted before and after treatment with ^{14}C-Glucose.

Sixty millilitre of ghosts were treated with 10 ml. glucose solution containing 20 µc total radioactivity and then treated with 330 ml. ice cold propanol for one hour and 210 ml. chloroform for a further hour before being filtered. If any complex were formed, the material should

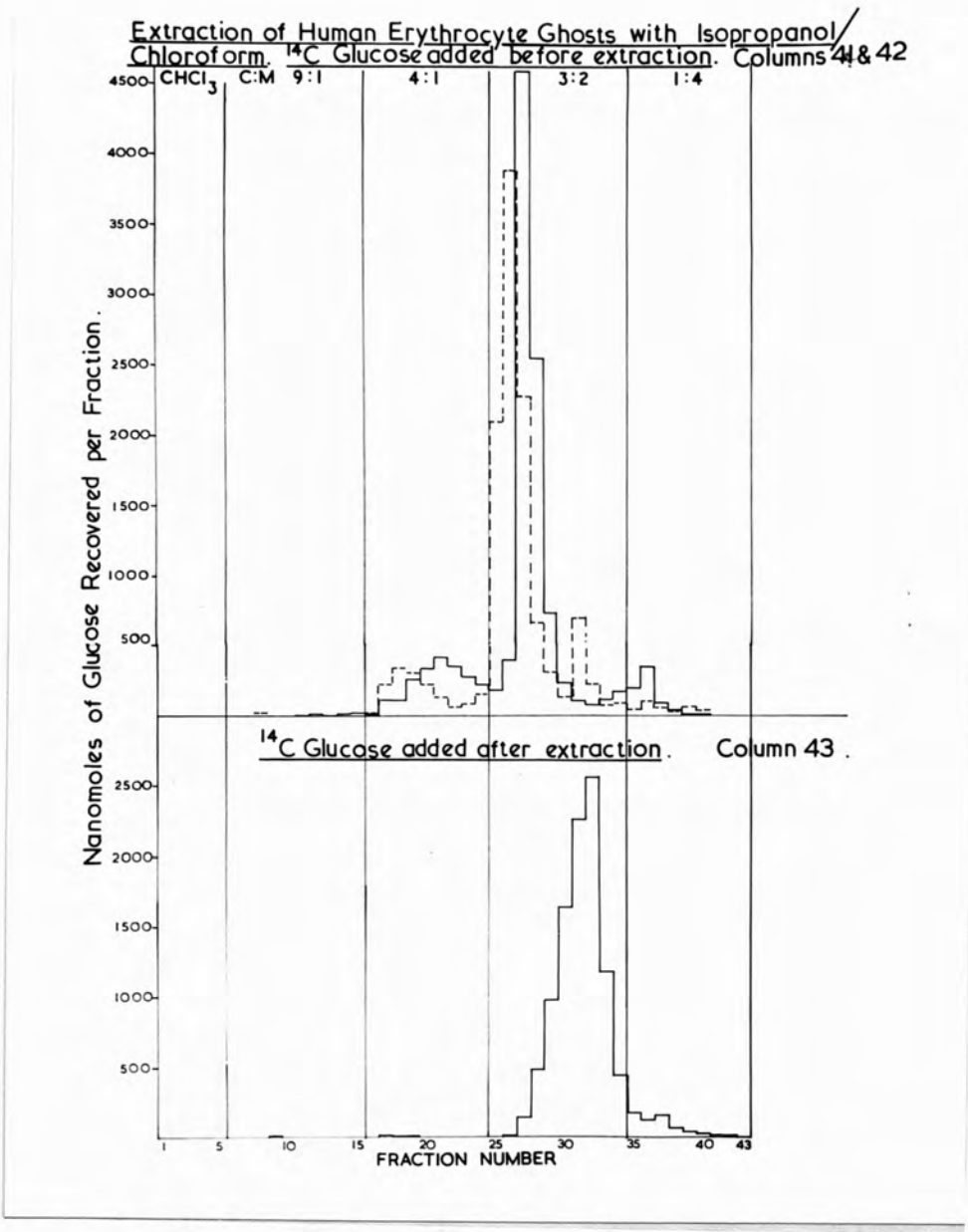


Fig. 10.14. - Elution pattern of glucose recovered in isopropanol: chloroform extract of human erythrocytes. ^{14}C-glucose was added 1) before extraction (columns 41 and 42) 2) after extraction (column 43).

be extracted in this way.

In the second experiment the glucose was not added until after treatment with isopropanol. Any complex formed in handling the material should appear in this experiment.

Columns run with this material gave promising results in that the early radioactive peak in chloroform:methanol 4:1 and the late peak in chloroform:methanol 1:4 already mentioned were obtained from material incubated with ^{14}C-glucose before extraction and absent from material when ^{14}C-glucose was added after. (see fig 10.4).

As it seemed possible that any complex might be broken down by the water in the solvent system used in developing the thin layer plates, several solvent systems were investigated. Dimethyl formamide alone seemed the most useful and thin layer separations using this solvent seemed to indicate that while the radioactive material in fraction 36 existed as free glucose, some of that in fractions 20 and 26 was bound, fractions 19, 22 and 23 were pooled and run in D.M.F. The radioactive material was eluted and shaken with ether and water when 60% of the counts were recovered in the ether. When run on plates, the chief material seemed to be lysophosphatidyl ethanolamine.

The remainder of the red cell extract ~~extract~~ of experiment 1, which had been stored at 4°C in an atmosphere of nitrogen was fractionated on a silicic acid column no. 42 giving elution curves very similar to those already described. Fractions 18 and 33, from the early and late radioactive peaks were run on plates.

It seems that glucose incubated with human erythrocytes is able

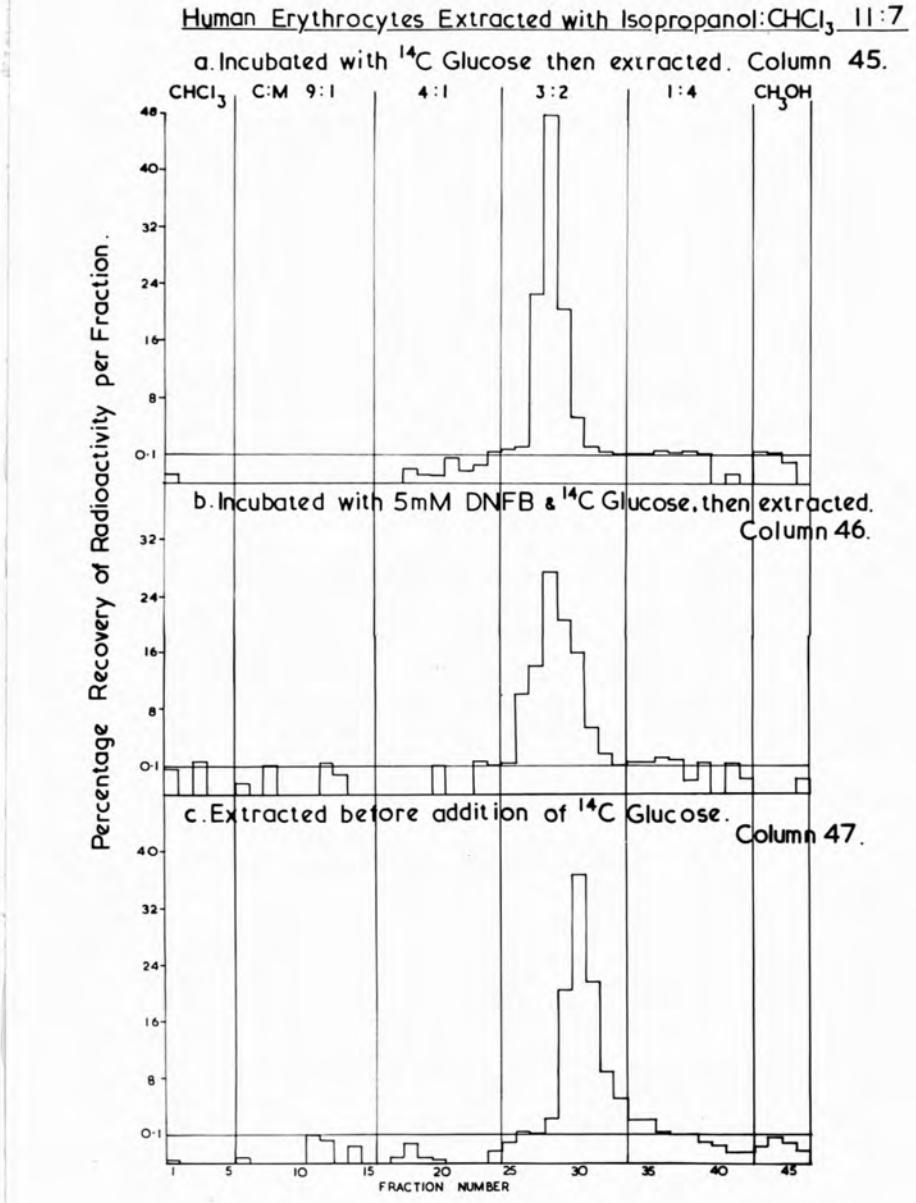


Fig. 10.15. - Elution pattern of glucose recovered in isopropanol: chloroform extracts of human erythrocytes.

cells incubated with a) ^{14}C-glucose (column 45)

b) 5 mM DNFB and then ^{14}C-glucose (column 46)

c) ^{14}C-glucose after extraction (column 47)

to combine with some component in the ghost. This association is sufficiently stable in isopropanol:chloroform for it to be extracted in this solvent. It could also be recovered from a silicic acid column. The suggestion that glucose was bound was to some extent confirmed by thin layer chromatography and solvent partition experiments.

Uptake of Labelling by Whole Red Cells incubated with ^{14}C-Glucose and Extracted with Isopropanol:Chloroform 11:7.

It seemed that a type of lipid glucose complex could be extracted from human erythrocyte ghosts using isopropanol:chloroform. It was, therefore, decided to repeat the experiments using intact erythrocytes to see if a similar complex could be obtained from unhaemolysed cells. Extraction was also performed with cells treated with DNFB as an indication of whether the material was implicated in glucose transfer. In experiment A 100 ml. packed red cells were incubated with 7 ml. ^{14}C-glucose solution - total activity 14 µc, and then extracted with 415 ml. ice cold propanol and after one hour with 265 ml. chloroform. In experiment B 150 ml. cells were incubated for one hour in 1 litre saline buffer containing 5 mM DNFB and 100 ml. of inhibited cells were treated with 7 ml. ^{14}C-glucose solution and extracted as in A. Experiment C was again similar to experiment A but the glucose was added after extraction with isopropanol as in the previous experiment using ghosts. The solvent in each case were decanted one hour after the chloroform had been added and the remaining material dried and re-extracted with warm solvents, but no further significant quantity of radioactivity was obtained.

Unfortunately column chromatography failed to reveal the two smaller radioactive peaks on either side of the free glucose peak

seen in the columns run of the isopropoanol:chloroform extraction of erythrocyte ghosts. The radioactivity in the three peaks from the three experiments performed with whole cells run as free glucose on a plate developed with DMF.

The failure to obtain radioactive peaks other than that of free glucose was unlikely to be the result of variations in the technique used, but could result from exposure of reactive site during the preparation of ghosts. Extraction of ghosts containing ^{14}C-glucose was later repeated in this laboratory using exactly the same method and the material obtained was divided into two fractions, that soluble in chloroform and that in DMF. The two small radioactive peaks seemed to be associated with the DMF soluble material.

Treatment of Human Erythrocytes with Sodium Boronhydride.

While there was evidence of glucose lipid complexes in the extraction carried out above, only limited treatment was possible owing to the apparent instability of such complexes. As a more stable glucose complex would materially assist in the extraction procedures, attempts were made to see if this could be obtained. More stable binding of glucose was not obtained by the use of DNFB so that experiments were carried out to see if the glucose complex could be affected by reagents capable of stabilizing temporary chemical structure, such as Schiff's bases. Sodium boronhydride has been used to stabilized osomethine linkages and Fischer et al. (1955, 1962) applied to this reagent to a phosphopyridoxal protein system and in 1964 used it in experiments on binding of vitamin B6 co-enzymes and further labelling of the active site of enzymes

Human red cells, equilibrated with 76 mM glucose, were incubated with sodium boronhydride in the range of concentrations 0.025 - 0.25 M for periods of time varying from 5 mins - 6 hours. The cells were then spun off and glucose exits and entries tested at 27°C, 22°C and 16°C. Some clumping of the cells was found and after incubation for longer periods with concentrations of sodium boronhydride above 0.1 M haemolysis was found to occur. Little effect on glucose exit or entry was found as would be expected if the reagent were making the link between the membrane component permanent. The red cells were further incubated with sodium boronhydride and exits into 21 ml. saline containing 0.5 mg sodium boronhydride at pH 5.4, 5.7, 6.0, 7.0, and 8.0 were followed in the presence of varying concentrations of glucose. Entries were also followed. Again no significant change could been seen.

Urea, since it is able to break hydrogen bonds could facilitate the action of sodium boronhydride and, therefore, the effect of sodium boronhydride was examined after the cells had been treated with 0.3 M urea, again with negative results. Treatment of red cells with lithium boronhydride in the presence of glucose also proved to have no effect on the kinetics of transfer.

CHAPTER 11.

Discussion.

Transport problems are occupying the attention of physiologists in many fields at the present time. A full understanding of permeability, so essential for an insight into numerous mechanisms, from the action of hormones at one extreme to the generation of potentials in nerve and muscle at the other, has not yet been achieved. As described in Chapter I, a considerable amount of work has been done on the nature of the plasma membrane, a knowledge of which is basic to any understanding of permeability, but as yet no entirely satisfactory picture seems to have been produced. There seems to have been a tendancy to regard the plasma membrane as a rather rigid structure which may have introduced difficulties into the consideration of many membrane phenomena.

Recent work on the passage of water through artificial phospholipid membranes, however, assumes the lipid interior to be in a liquid state (Hanai and Hayden, 1966), as does the work of Luzzati et al. (1966).

In considering the penetration of both electrolytes and certain non-electrolytes such as sugars into a wide variety of tissues, the mechanism adopted by most authors as a result of a strong body of experimental evidence (see for example Henderson 1964) is that of carrier transport. For this process (described in many review articles e.g. Wilbrandt, 1961; Widdas, 1963) a first essential step is a reaction between the substrate and a component of the cell membrane. The nature of this component is unknown but for sugars it is considered to be mobile within the membrane (Widdas, 1962

Rosenberg and Wilbrandt 1957). The nature of the reaction is also unknown, but the kinetics allow that the complex could be formed either with or without the mediation of an enzyme. In either case both the association and dissociation must be very rapid since the movement of the complex across the membrane is thought to be the rate limiting step.

The experiments described in this thesis were aimed to attack the problem on several fronts by following the uptake of inhibitors, and by studying their effect on the parameters of glucose transfer. and their possible binding to red cell lipids. The possibility of the binding of glucose to these lipids was also studied and extraction techniques developed to this end.

Kinetic Section.

Of the approaches used, those involving kinetic techniques yielded some interesting information. The extraction and isolation experiments failed to give any very conclusive results. A biochemical description of the processes involved in facilitated diffusion has for some time been seen to be very elusive.

Studies on competitive inhibitors involved only the use of diphenols. Phlorrhizin is known to exert an inhibiting effect on glucose transfer in a wide variery of tissues, kidney, intestine, erythrocyte and rat heart muscle. It was originally believed that this compound has the same action in all these tissues, but the observation by Wilbrandt that the aglucone phloretin is many

COMPETITIVE INHIBITORS.

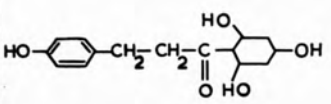
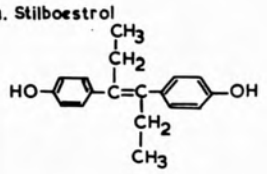
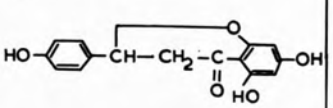
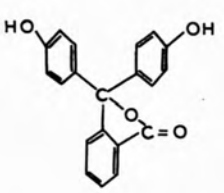
Inhibitor.	Formula.	Concentration for 50% Inhibition at 37°C. Expressed as reciprocals of Millimolarity. After LeFevre	By method of Sen and Widdas
1. Phloretin.		100	1.5×10^{-6}M
2. Phloretin Derivatives.	Not Diphenols	$\ll$0.1—7.0	
3. Synthetic Oestrogens.	a. Stilboestrol 	160	2.5×10^{-6}M
	b. Naringenin 	7	
4. Phenolphthalein			$20-25 \times 10^{-6}$M

Table 11.1. - Showing structure and inhibitory potency of diphenolic competitive inhibitors.

times more potent in the erythrocyte casts doubt on this assumption. Laris et al. (1961) found that phloretin and phlorrhizin are inhibitors of stromal ATPase but concluded that the ATPase activity is not essential for sugar permeability. LeFevre (1959b) has studied the action of many derivatives of phloretin and found that derivatives in which the hydroxyl group had been methylated were relatively ineffective and that there was an increase in potency with an increase in the size of the aliphatic side chain. He also found that break-down products of phloretin were relatively inert. The chief compounds used in the present studies were phloretin, stilboestrol and phenolphthalein, whose structures, may be seen in table 11:1. In phloretin which differs from the other two compounds in that one benzene ring bears three hydroxyl groups, the rotation of the two rings relative to one another is not restricted, and the two terminal hydroxyl groups can approach quite close to one another. The structure of stilboestrol and phenophthalein is more rigid. In stilboestrol, where the aliphatic chains are present, full extension is maintained between the two hydroxyl groups. Studies with the two latter compounds revealed that they were true competitive inhibitors and that their action was not dependent on pH.

Phloretin appeared to be the most potent inhibitor. The concentration of this inhibitor required to double the uninhibited exit time (half saturation constant for the inhibitor) at 37°C was found to be 1.5×10^{6}M. Stilboestrol was nearly as effective 2.5×10^{6} being required for 50% inhibition at 37°C. This is the

reverse order to that found by LeFevre. An explanation for this may be found in the fact that different cell concentrations were used, and due to a high cell-water partition the resulting water concentrations are reduced. Phenolphthalein was some twelve times less effective than phloretin, a concentration of between 20 and 25 x 10^6 being required to produce 50% inhibition. This still represents a very low concentration of inhibitor and phenolphthalein may be regarded as quite an effective inhibitor of glucose transfer.

With all these inhibitors it was found that, as for glucose (Sen and Widdas 1962a) the dissociation equilibtrium was a continuous function of temperature between 10° and 40°C and it would appear that the breakdown of complexes formed with the membrane component envisaged as mediating in the transfer process requires more energy than that for their formation. The differences in these two energies appeared to be greater for phloretin and phenolphthalein than for glucose and presumably this is one reason why these inhibitors are effective at such low concentrations. The slope of the Arrhenius plot of $\emptyset_I$ for stil boestrol is very shallow, 4,600 cal/mole maximum as opposed to 10,000 cal/mole for glucose. The reason for this is not apparent and in this case a meaningful correlation between chemical structure and activity is difficult to make. There are several reactions to be taken into account including competition with glucose.

The experiments with phenolphthalein which have been briefly reported (Forsling & Widdas, 1965a, see Appendix ii) and (Forsling & Widdas, 1965b) were of special interest

since this reagent had not been used previously as an inhibitor of glucose transfer. Subsequently Sandford and Smyth (1966) investigated the effect, on intestinal transport of glucose, of several polyphenolic compounds including phenolphthalein and phenol red. They found that phenolphthalein was nearly as effective as phlorrhizin in inhibiting transfer, although the effect was less specific, while phenol red in concentration of 10^{-4} M had little effect. They suggested that this difference in activity could be due to the fact that at the pH used, 7.4, phenolphthalein was mainly undissociated while phenol red was largely dissociated. This inhibition of glucose transfer by phenolphthalein may be related to its action as a cathartic. Love, Mitchell and Phillips (1965) have found that phenolphthalein amongst other cathartics is effective in inhibiting sodium transfer across frog skin and in the small intestine of rabbit. The classical view of the action of phenolphthalein is that it stimulates the smooth muscle of the intestine directly casuing increased motility of the muscle. In the light of these observations it seems that this may not be the case. It is possible that phenolphthalein by affecting solute movement also affects the reabsorption of water and acts in this way.

Amongst those compounds found to be competitive inhibitors of glucose, those most widely studied have been mercuric salts, organic mercurials and the protein reagents 1 - fluoro-2, 4-dinitrobenzene (DNFB) and N-ethylmalemide (NEM). The mercuric compounds give an inhibition which can be reversed by adding excess of a

compound containing a thiol group, for example cysteine but DNFB and NEM are irreversible inhibitors since they react to form covalent bonds. The action of the related compounds 1, 5-difluoro-2, 4-dinitrobenzene (FDNFB) and N-phenyl maldmide (NPM) were studied here to see if they could provide more specific inhibitors than those hitherto used. The kinetics of inhibition of DNFB had been previously studied by Bowyer and Widdas (1956, 1958) and those of NEM by Dawson and Widdas (1963). The action of both compounds was found to be very dependent on temperature. The inhibition was found to be rapid at first and then to slow down until approximately 97% inhibition had been achieved, but did not correspond to a first order reaction. Dawson and Widdas found that the reaction could be quite fairly described using the two first order equations, one about six times faster than the other. They also found it necessary to assume that for NEM a small proportion of the sites was un-reactive. The pattern of development of inhibition of FDNFB and NPM was found to be essentially the same as that described for DNFB and NEM and the mechanism of action of the two former compounds seemed to be rather similar. The two inhibitors studied did not appear to be in any way superior to those previously used.

Berg et al (1965) have described the action of FDNFB. They were not able to follow the movement of non-electrolytes as they found the membrane structure became too rigid. In the present studies glucose movement was conveniently followed in the photo-electric apparatus. It was only after five hours that no volume change

could be observed, presumably as a result of internal links being formed in the membrane. It was, however, still possible to obtain haemolysis by placing the cells in distilled water. Studies with ^{14}C-glucose confirmed that glucose could penetrate cells treated with FDNFB. One interesting point arising from studies with this inhibitor was that the slope of the Arrhenius plot of the half saturation constant for glucose was reduced in cells where transport was 90% or more inhibited. This suggests that the mechanism of residual permeability might differ from that normally in action

FDNFB treated cells proved to be useful material for the study of exchange diffusion as exit times were considerably increased. Movement of labelling from inhibited cells loaded with ^{14}C-glucose into a saline medium containing 76 mM glucose, which occured as a result of exchange of complexed labelled glucose for unlabelled glucose in the external solution was found to occur at a faster rate than the movement of glucose from loaded cells into saline. These results were taken to confirm the work of Britton (1964), and Mawe and Hempling (1965), who showed that bound carrier has a higher rate of migration than the free carrier. This observation would tend to discount the possibility that glucose passing across the membrane exchanges with a sugar moiety of some membrane component. Some degree of uncertainity was introduced, as to the time for glucose to leave the cells, since four minutes was required to spin down the cells. It was proposed to overcome this by separating the cells

from the plasma by ultra-filtration. Unfortunately time did not permit these experiments to be carried out.

The brief studies with dexoxyribose also demonstrated that there are methods other than carrier transport for sugar penetration. The results from experiments using sodium free media served to re-affirm that sugar transport in human erythrocytes is independent of sodium and likely to be independent of energy sources. This was shown to be the case with time expired cells. Glucose transfer was found to be little impaired in these cells which are virtually depleted of energy sources.

In addition to studying the action of non-competitive inhibitors, attempts were made to correlate the uptake of inhibitor with the development of inhibition. ^{14}C-labelled NEM and DNFB were used to this end. Those experiments using DNFB were more successful than those carried out with NEM. The relationship between inhibition and uptake was found, for DNFB, to be independent of temperature. For both compounds a sigmoid relation was found between uptake and degree of inhibition. Over one part of the curve a large increase in percentage inhibition was found for a relatively small uptake of material. From this steep part of the curve a figure was derived of the maximum inhibitor uptake necessary to give 100% inhibition. This was 1×10^9 molecules per cell for NEM and 4×10^8 for DNFB. This method of determining the maximum possible sites available was quite valuable as kinetic studies alone give very little information

on this point since they do not distinguish between a large number of components acting slowly and a few components with a very fast glucose turnover. The assumption that DNFB is reacting with a site involved specifically in glucose transfer and has not a non-specific manner such as stiffening the membrane seems justified in the light of observations made with malonamide in DNFB treated cells.

The figure obtained of 4×10^8 molecules per cell to give 100% inhibition is obviously an overestimate as there are numerous other sites not concerned with transport in the cell with which DNFB is free to react. Morell et al (1964) and Weed et al. (1962, 1966), have shown that the majority of these reagents would be taken up by the haemoglobin. LeFevre et al (1959) found an uptake of phloretin of 50×10^6 molecules per cell for 50% inhibition. Again much of this represented non specific uptake. Weed, Van Stevenick and Rothstein (1964) using mercurial compounds found that glucose transfer was inhibited when 1.2% of the membrane SH groups had reacted with parachloromercuribenzoate (PMCB) and from this they calculated that the maximum number of transport sites in the red cell would be 700,000 and that this number of glucose molecules would cover about 1% of the red cell surface.

LeFevre, however, in 1961(b) using a method of incubating ghost suspension in low concentrations of glucose and spinning them calculated that the number of sites concerned in glucose transfer could not be greater than 500,000 per cell and assumed that the

results for the intact red cell would be similar. This figure seems rather low and it may be that such results obtained with ghosts cannot be directly applied to whole red cells.

As regards the uptake of competitive inhibitors, it was not possible to demonstrate any relation between concentration of glucose present and uptake of inhibitor. (Uptake experiments of the type used for non-competitive inhibitors were not possible.) This was shown to be a result of partitioning of the inhibitor stilboestrol between the cells and the medium in the ratio of 115:1. This value was obtained on the assumption that stilboestrol penetrates the cells. LeFevre et al. (1959) found that they could not say if this was actually the case. If stilboestrol passed into the membrane only, a distribution ratio up to many thousands could exist. It would be interesting to see the partition ratio of stilboestrol between a lipid extract and a solution of haemoglobin.

LeFevre et al. working on gross uptake of inhibitors found similar results for several competitive inhibitors, quoting a distribution ratio of 50 or more. These authors found a parallel between the effect of pH on gross attachment and its effect on transport inhibition. They also found no evidence of saturation of uptake even at the highest concentrations attainable, although the inhibitory kinetics would imply saturation of the sugar transport sites at much lower levels. In view of this non-specific uptake they suggested than an overestimate of the half saturation constant

for some of the more potent inhibitors could have been made. The reverse, in fact, could be the case. If stilboestrol was concentrated in the membrane, and was an effective competitor in that phase, the actual concentration reacting with the membrane sites may, in fact, be comparable to that for glucose.

Biochemical Section.

Little evidence is available as to the particular biochemical nature of the component involved in transport although the list of requirements grows increasingly long. On the whole lipids were thought to fulfil these better than other group of compounds. Phosopholipds, as relatively small molecules with a variety of active "head" groups are suitable candidates. That combination with a non-protein molecule facilitates the passage of glucose across the membrane has been shown by Rosenberg and Wilbrandt who found that whereas glucose could not penetrate cattle erythrocytes glucose benzoate could. The experiments of Hillman et al. (1964) lend support to the hypothesis of a lipid substance as a controlling factor. They found that an increase in the length of the side chain in the substrate sugar molecule from methyl to butyl led to an increase in the rate of equilibration of sugar between extracellular and intracellular water. In the nineteenth century it was thought that a definite family of compounds was formed by the reaction of sugars with phosopholipds. They were called "jecorines". Recently the

suggestion of such a complex came from Park. He reported in 1961 that Reinwein had been able to solubilize glucose in chloroform with a small quantity of lipid extracted from the erythrocyte ghost. It was shown that this was not due to the trapping of water by molecules. LeFevre et al. (1964) made lipid extracts of red cells and ghosts in the presence of ^{14}C-glucose and found that by extracting with hot methanol-ether they were able to obtain an extract with up to 5×10^8 molecules of glucose per ghost. The glucose could be recovered by shaking with water. Further support that lipid or lipid soluble complexes are involved in the facilitated transfer mechanism comes from the work of Reiser and Reiser (1964) on insulin stimulation of sugar transport in red cells treated with chymotrypsin. They found the effect could be abolished by phospholipase C suggesting that lecithin was implicated in insulin facilitated transfer. There is, however, a growing body of evidence in favour of protein if not enzyme involvement. For example a very recent paper by Kennedy et al. proposes a convincing transport system for lactose in E. Coli involving a protein carrier.

In the field of electrolyte transfer there is again considerable evidence of lipids participating as carriers. The work of Hokin and Hokin (1964) seems to favour phosphatidic acid and that of Dawson and his colleagues triphosphoinositide. Phospholipids are an obvious choice for this role as their polar groups normally reside on the outside of the membrane and could attract charged ions. Solomon et al. (1956) studied various phosphatidyl fractions and found that

the fraction containing phosphatidyl serine had the greatest ability to distinguish between Na^+ and K^+. Phosphatidyl serine was actually suggested as a possible Na^+ carrier by Kirschner in 1958 as a result of work on the cation content of phospholipids from swine erythrocytes. Watkins considering the pharmacologlical receptors and transport, discussed the possibility of a permeating molecule exchanging with a moiety of a membrane component in the course of crossing the membrane. It is tempting to suggest that glucose could enter by exchanging with the sugar group of some membrane component although results from extraction and uptake experiments do not tend to confirm this.

When the present work on the extraction of phospholipids from red cells began, the literature was not so copious or informative as it is now so that much of the work was concerned with the development of techniques applicable to the problem in hand. Early experiments seemed to indicate that butanol was a more efficient solvent than the conventional chloroform:methanol since it yielded good recoveries of both radio active labelling from glucose and lipid. It is able to release lipid from association with protein as described by Maddy (1964). A single phase system was also more desirable than one such as chloroform:methanol where it was felt that much material of interest, might be tending to accumulate at the interface between chloroform and the aqueous phase and be difficult to separate. Subsequent work on the attempted isolation of a glucose-lipid complex using butanol to extract the erythrocyte ghosts failed to give any very significant results so that other solvents were used. Of these isopropanol: chloroform seemed to give

quite promising results.

It was hoped originally that sufficient information would be provided by studies on inhibitor binding so that lengthy pursuance of the question of lipid binding of glucose would not be necessary. The use of ^{14}C-glucose in this connection presents several obvious problems and involves the introduction of certain assumptions. Glucose is to a some extent soluble in phospholipids so that many controls are necessary. Fundamental to the concept of transport is the proposition that the action leading to the formation of the hypothetical complex is easily reversible, so that the complex is of necessity unstable. The first factor makes it difficult to distinguish between the appearance of ^{14}C-labelling in a fraction as the result of solubilization and as a result of true complexing of glucose with carrier. The second makes treatment of complexed material hazardous and renders questionable the use of standard techniques of lipid chemistry. Another danger - metabolic incorporation of glucose was reduced by using short incubation times, low temperatures and in some cases erythrocyte ghosts. This last method, however, introduces a further small degree of uncertainity in that the transfer mechanism may not be entirely functional in ghosts. There is, however, considerable evidence that the mechanism is intact and little evidence to the contrary.

In spite of these difficulties it was felt that this study was useful and at the present time seemed to present one of the few approaches that had not been extensively studied and could yield valuable information. It should be emphasised that the primary aim of these experiments involving solubilization and separation of

membrane components was to determine if ^{14}C-labelling from any of the compounds used, either inhibitors or sugar, could be incorporated into any lipid fraction of the membrane material. It was hoped, however, that some indication of the nature of the fraction would be obtained from its chromatographic behaviour.

A protein material which separated from the lipid extract was found in many cases to be associated with ^{14}C-labelling. Unfortunately time permitted only scant examination of this material. There was also the difficulty of working with the material since only the labelling with protein reagents could be expected to remain attached to any protein moiety treated with aqueous solvents.

The biochemical aspect of transport has proved and is still proving to be a difficult problem. Therefore the several lines of attack which presented themselves were followed. The first and most obvious opening to a solution of the problem seemed to come from stable marking of the membrane component involved with a radioactively labelled non-competitive inhibitor. The results of uptake experiments using ^{14}C-Nem and ^{14}C-DNFB led to their use in experiments in which marking of red cell lipids was attempted.

It was found possible to incorporate ^{14}C-labelling from DNFB into lipids of the red cell extracted with butanol. Of the labelling added 1% could be detected in butanol soluble lipids. This material when subjected to column chromatography exhibited different behaviour from free DNFB. Of the ^{14}C-DNFB recovered in the lipid fractions 2% was obtained in the fractions where phosphatidyl ethanolamine

was eluted. This compound is not necessarily involved since DNFB, by blocking the reactive head group, probably reduces adsorption of the lipid on the silicic acid.

If results from experiments with sheep erythrocytes are compared with those in which human erythrocytes were used it is found that the label in this fraction in lipid from human erythrocytes exceeds that in sheep erythrocytes by a figure which is 10% of the total counts and represents one million molecules of DNFB per red cell. This exceeds the possible estimate for the number of sites. This combined with the fact that DNP ethanolamine could be recovered from hydrolysates of lipid associated with ^{14}C label is suggestive that this compound could in some way be involved in transport. Other molecular groups found to bear DNP labelling were lysine and possibly leucine and glycine. Berg et al. (1965) claim to have found that the group with which DNFB reacts in the membrane is the ϵ amino group of lysine. Other workers, who have used DNFB to determine which membrane component is involved in transfer, are LeFevre et al. (1964) and Stein (1964). LeFevre et al. found that their recovery of glucose in lipids extracted from red cells with hot methanol-ether was reduced by two thirds if the cells were incubated with DNFB before the glucose was added. Stein claimed to have isolated a protein component from the membrane which could be involved in transport. DNFB at high concentrations reacts with the membrane in a fashion dependent on the square of the concentration. Using different concentrations of DNFB labelled with ^{14}C and ^{3}H, he attempted to separate the component which reacts in the membrane.

DNFB will attack not only thiol groups but also amino groups and other hydroxyls such as phenolic hydroxyls. One might think that NEM could be more specific so that the effect of ^{14}C-NEM was investigated along the same lines as DNFB. Even with this inhibitor labelling was found in the butanol soluble lipids obtained from human erythrocytes and human erythrocyte ghosts. The experiments carried out gave little indication of the chemical compounds carrying NEM label and unfortunately the lack of supply of stable ^{14}C-NEM prevented further investigation.

The competitive inhibitors such as phloretin, phenolphthalein and stilboestrol are very effective in low concentrations so that the use of these compounds might result in a more specific labelling of material participating in glucose transfer. Experiments with phenolphthalein and ^{14}C-stilboestrol were performed to this end. Labelling from ^{14}C-stilboestrol did appear in several lipid fractions separated from a silicic acid column and when these fractions were run on thin layer plates the radioactivity was found to be distributed differently from free stilboestrol. The quantities recovered, however, were not sufficiently large to warrant extension of studies using this material and in view of the high distribution ratio of stilboestrol between cell and medium, this may not be the best material with which to work. Polyphloretin phosphate which does not penetrate the cell and would be expected, as a result of the phosphate groups, to have a reduced lipid solubility might give stable marking

of the glucose binding component in the erythrocyte membrane. If polyphloretin phosphate becomes available, isotopically labelled, experiments with this material on lines similar to those with stilboestrol might prove quite profitable.

It was found possible to obtain a quantity of ^{14}C-labelled glucose associated with lipids extracted with butanol. This material was felt to be of some significance in that it was consistently obtained. It represents only a small percentage of the total radioactivity present, some 6 - 18%, but would be sufficient to account for labelling of sites of glucose binding. There is good reason to believe that this glucose was bound since it chromatographed differently from glucose, coming off the column in chloroform:methanol 4:1 and 1:4 when free glucose appears in chloroform:methanol 3:2. On thin layer plates developed in chloroform: methanol:water 80:30:3, this radioactivity was associated with lipid spots of Rf approximately 0.3 and 0.6. Free glucose remained at the origin. Part of the radioactivity appearing in chloroform: methanol 4:1 could be due to trailing of glucose in the presence of lipids. It is not possible to say with which lipid or lipids the radioactivity was associated, but it appeared in fractions where phosphatidyl ethanolamine, sphingomyelin and lyso-lecithin are eluted.

The material of the acid butanol extraction eluted with chloroform:methanol 9:1 from silicic acid columns seemed at first to be of some interest, but successive experiments using increasing

concentrations of glucose, DNFB and phloretin treated ghosts, and sheep erythrocyte ghosts, all of which failed to affect the quantity of this material recovered, indicated that this material was not of biological interest. It does, however, indicate that glucose readily reacts with compounds which render it lipid soluble. The material formed behaved in the manner of a lipid on chromatography and can be compared with the experiments of Rosenberg and Wilbrandt who found that the formation of glucose benzoate from glucose resulted in the glucose acting as a lipid soluble molecule. The compound formed in these experiments thought to be butyl glycoside, was stable so that ^{14}C labelling was not freed during chromatography as described by Lefevre (1963). Thus the developing solvent chloroform:methanol:water 80:30:3 used for thin layer chromatography in experiments involving ^{14}C labelled inhibitors seemed satisfactory. Other non-aqueous solvent systems had, however, to be examined for use with extracts, containing ^{14}C-glucose, obtained with such solvent systems as isopropanol:chloroform.

A small but constant amount of radioactive glucose was eluted, in association with lipids, from silicic acid columns in chloroform: methanol 4:1 and 1:4 in experiments where erythrocyte ghosts were incubated with ^{14}C-glucose before extraction with isopropanol. This was not found in extracts where glucose was added after extraction, so it cannot be a product of the preparative procedures. Further tests indicated that glucose was in fact bound to material which rendered it soluble in many lipid solvents. On thin layer chromatograms

developed with DMF the radioactivity stayed with the lipids. The distribution of labelling between ether and DMF was found to be 0.075 for fractions eluted from the column in chloroform:methanol 4:1. Free glucose distributed itself almost entirely in DMF. Unfortunately it was not found possible to obtain this material from whole cells. Extraction of ghosts containing ^{14}C-glucose was later repeated using the same method and the material obtained was divided into two fractions, that soluble in chloroform and that in DMF. The two small radioactive peaks, obtained in chloroform:methanol 4:1 and 1:4, seemed to be associated with the DMF soluble material.

As a more stable glucose complex would materially assist in the extraction procedure, experiments were carried out in an attempt to render the assumed link more stable. If DNFB were acting by super-imposing itself on glucose, as has been suggested, treatment with this reagent could lead to a more permanent binding of glucose. This was not found to be possible. Attempts were also made to see if the glucose complex could be affected by reagents capable of stabilizing temporary chemical structures such as Schiff's bases. Treatment of red cells in the presence of glucose by sodium boronhydride and lithium boronhydride proved, however, to have no effect on the kinetics of transfer.

Conclusion and Points for future work.

Although the biochemical experiments yielded relatively little information as to the possible nature of the postulated carrier,

they covered quite a wide ground and laid a reasonable foundation for further work. It seems likely that extraction and separation carried out on lines similar to those described with perhaps variations in solvents and adsorbents would give valuable results. Other possible lines of approach include selective removal of components from the membrane as described by Parpart and Bellentine (1952) and Van Dennen and De Gier (1964) for red cells and Dikstein and Selman (1965) for frog rectus abdominus muscle and a simultaneous study of the resulting permeability changes, assuming they were not too gross. Experiments of possible interest could be the study of glucose permeability of artificial membranes, such as used by Hanai and Taylor and Huang and Thompson, or spherulites as described by Bangham et al. (1965) and Weissman et al. (1965). A systematic study of changes in composition and permeability could prove interesting. However, since the carrier molecule is assumed to be present in relatively small amounts it might be that all these approaches are rather like trying a delicate dissection with a large knife.

Finally, although interest in this work has centred on lipids the possibility of protein involvement has to be considered. While this script was in preparation work has been published by Bobinski and Stein (1966), and Bonsall and Hunt (1966) implicating proteins. Their criterion for glucose binding, as has been to a large extent in this thesis, is altered chromatographic behaviour, although they used a novel system - columns pre-loaded with membrane extract.

Isolated components were used so that there can be no certainty that the material holding glucose back on the column is involved in transport. As they used whole cells and extracts prepared with Triton X100, the possibility of lipid participation is not entirely excluded.

SUMMARY.

1. A brief history is given of the development of the concept of a plasma membrane, with special reference to the erythrocyte membrane. The methods available for studying membranes are discussed and the current ideas regarding membrane structure are presented.

2. The present position with respect to glucose transfer across the erythrocyte membrane is reviewed and several approaches to the problem proposed.

3. The action of competitive inhibitors on glucose transfer was studied. Phenolphthalien is shown to be an inhibitor of glucose transfer. Its action is competitive and the slope of the Arrhenius plot (log $\emptyset_I$ against 1/T) is found to be 19,000 cal/mole. That for phloretin was 20,000 cal/mole indicating that for both compounds there is a greater difference between the energy required for formation and dissociation of complexes than for glucose. An attempt was made to relate the results to the structure of inhibitors.

4. Stilboestrol was also shown to be a competitive inhibitor. The shallow slope of the Arrenhius plot 4,600 cal/mole is suggestive that lipid solubility or conformational changes with temperature are occurring.

5. The action of N)phenyl maleimide and dinitrodifluorobenzene (FDNFB) was investigated and the course of inhibition found to be similar

to that for N-ethyl maleimide (NEM) and dinitrofluorobenzene (DNFB). FDNFB treated cells were used to follow exchange diffusion with results which are consistent with the view that the rate of movement of the bound carrier is greater than that of the free carrier.

6. Studies using galactose indicate that the galactose carrier complex dissociates more readily than the glucose carrier complex, and studies with 2-deoxyribose suggest that penetration of this compound is by a different system from that of glucose.

7. Experiments, using modified suspension media, indicated that alterations of Na^+ concentration do not affect glucose transfer in the erythrocyte.

8. The uptakes of ^{14}C-labelled inhibitors were studied. Stilbeostrol distributes between cells and medium with a ratio of 115:1. Uptakes of NEM and DNFB by red cells were measured and calculated to be 1×10^9 and 4×10^8 molecules per red cell respectively to give full inhibition of transfer. This is assumed to be an overestimate of the number of carrier sites in the membrane.

9. A description is given of the methods evolved for extraction and separation of phospholipids from erythrocytes after incubations with isotopically labelled inhibitors and isotopically labelled glucose.

10. Experiments involving isolation of lipids from cells incubated with competitive inhibitors yielded little information of

interest, but with the irreversible inhibitor DNFB labelling could be detected in butanol soluble lipids. A considerable part of the labelling was attached to phosphatidyl ethanolamine. Caution must be exercised in interpreting results obtained from isolating radioactive-labelled cellular components owing to the fact that the kinetics of reaction of DNFB are complex and other lipids were labelled.

11. It was found possible to obtain ^{14}C-glucose in certain lipid fractions from cells extracted with butanol, but attempts to eliminate this using inhibitors ^{12}C-glucose were unsuccessful. Such labelling was also obtained in lipid extracts from sheep cells.

12. Labelling present in the acid butanol fractions were shown to be at least in part an artefact of the extracture procedures.

13. Using isopropanol:chloroform it was possible to recover ^{14}C-glucose, apparently complexed to erythrocyte lipids but further work is needed before it can be said that this is pertinent to the transfer process.

14. The results are discussed in relation to the present state of permeability and pointers to further work are considered.

REFERENCES.

Abderhalden, E. (1898). Zur quantitative verleich. Analyse des Blutes. Z. physiol. chem., 25, 65-115.

Adam, N.K. & Jessop, G. (1928). The structure of thin films Part XII Cholesterol and its effect in admixture with other substances. Proc. R. Soc. B., 120, 475-483.

Altman, K.I. (1953). The "in vitro" incorporation of α-C^{14}-acetate into the stroma of the erythrocyte. Archs. Biochem. Biophys., 48, 478-480.

Althausen, T.K. & Stockholm, M. (1938). Influence of the thyroid gland on absorption in the digestive tract. Am. J. Physiol., 123, 577-578.

Althausen, T.K. (1949). Hormonal and vitamin factors in intestinal absorption. Gastroenterology, 12, 467-480.

Ansell, G.B. & Hawthorne, J.N. (1964). Phospholipids: Chemistry, Metabolism and Function. B.B.A. Library, Vol. 3 Elsevier Publishing Co.

Axelrod, J. Reichentahl, J. & Brodie, B.B. (1953). The direct determination of phosphatidyl ethanolamine and phosphatidyl serine in plasma and red blood cells. J. biol. Chem., 204, 903-911.

Bach, J.S. (1939). The effect of phloridzin on carbohydrate metabolism "in vitro". Biochem J., 33, 802-810.

Bang, O. & Ørskov, S.L. (1937). Variations in the permeability of the red blood cells in man, with particular reference to the conditions obtaining in pernicious anemia. J. Clin. Inves., 16, 270-288.

Bangham, A.D. & Horne, R.W. (1962). Action of saponin on biological cell membranes. Nature, Lond., 196, 952-955.

Bangham, A.D. & Horne, R.W. (1963). The physical structure of phospholipids in salt solution in "metabolism and physiological significance of lipids". ed. R.M.C. Dawson London - Wiley.

Bangham, A.D. & Horne, R.W. (1964). Negative staining of phospholipids and their structural modification by surface-active agents observed in the electron microscope. J. molec.Biol. 8, 660-668

Bangham, A.D., Standish, M.M. & Weissman, G., (1965). The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. J. molec. Biol., 13, 253-259.

Barnett, R.J. & Ball, E.G. (1960), Metabolic and ultrastructual changes induced in adipose tissue by insulin. J. biophys. biochem. Cytol 8, 83-101.

Betraud, W.S. & Hedley R.H. (1963). Hexagonal patterns in cell membranes. Nature, Lond. 200, 89-90.

Berg, H.C., Diamond J.M. & Marsey, P.S. (1965). Erythrocyte membrane chemical modification. Science, N.Y. 150, 64-67.

Bjering, T. (1932). Studier over glucosens invandering minnesket rode - blodlegmer - Thesis Copenhagen - A Busch. Cited by Bang, O. & Ørskov, S.L. J. clin. Invest. 16, 279-285.

Blass, J., Routi, A., Leocomte, O & Macheboef, J.(1953) Recherches sur les constituants azotes des extraits lipidiques du sang (aminoacides, peptides, ethanolamine) Bull Spc. Chim. biol. 35, 959-968.

Bloch, K. (1960), (editor), Lipid Metabolism. John Wiley & Sons Inc., London.

Bobbitt, J.M. (1963). Thin-Layer Chromatography. Reinhold Publishing Corp. New York, Chapman & Hall Ltd., London.

Bobinski, H & Stein, W.D. (1966). Isolation of a glucose bindind component from human erythrocyte membranes. Nature, Lond. 211, 1366-1369.

Bonsall, R.W. & Hunt, S. (1966). Solubilization of a glucose binding component of the red cell membrane. Nature, London. 211, 1369-1370.

Booij, H.L. (1949). The protoplasmic membrane regarded as a lipoprotein complex. Proc. Faraday Soc. 6 , 143.

Bowyer, F. (1957). The kinetics of the penetration of non-electroyltes into the mammalian erythrocyte. Int. Rev. Cytol., 6, 469-511.

Bowyer, F. & Widdas, W.F. (1956). The factilitated transfer of glucose and related compounds across the erythrocyte membrane. Disc. Faraday Soc., 21, 251-258.

Bowyer, F & Widdas, W.F. (1958). The action of inhibitors on the facilitated hexose transfer system in erythrocytes. J. Physiol., 141, 219-232.

Brinkman, R. & van Dam.E (1920). Remarques sur la question de la repartition de la dextrose entre les globules rouges et le plasma. Archs. int. Physiol. 15, 105.-115.

Britton, H.G. (1962). Induced uphill transfer and downhill transport, relationship to the Ossing criterion. Nature, London. 198, 190-191.

Britton, H.G. (1964). Permeability of the human red cell to labelled glucose. J. Physiol, 170, 1-20.

Britton, H.G. (1966). Fluxes in passive, monovalent and ploy-valent carrier systems. J. theor. Biol., 10, 28-52.

Buchannan, A.A. (1960). Lipid synthesis by human leucocytes "in vitro". Biochem J., 75, 315-320.

Buugenberg de Jong, H.G. & Bonner J. (1935). Phosphatide auto-complex coascervates as ionic systems and their relation to the protoplasmic membrane. Protoplasma, 24, 198-218.

Bugenberg de Johng, H.G. & Saubert (1957). Fortschritte zum Thema der Modelle der Protoplasmamembrane Rolle des Cholesterins mit beteillgungaer Phosphatidosaüren bzw, Eiweistt nebern Phosphatiden. Protoplasma, 28, 353-359.

Canon, W.B., (1932). The wisdom of the body. Kegan Paul, Trench Trubner & Co. Ltd., London.

Chambers, R. (1922). A microinjection study on the permeability of the starfish egg. J. gen. Physiol., 5, 189-193.

Christensen, H.N. (1960). Reactive sites and biological transport. Adv. Protein Chem., 15, 239-341.

Clausen, T. (1965). The relationship between the transport of glucose and cations across cell membranes in isolated tissues. I. Stimulation of glycogen deposition and inhibition of lactic acid production in diaphragm induced by ouabin. Biochim. biophys. Acta., 109, 164-167.

Cohen, G.N. & Monod, J. (1957). Bacterial permeases. Bact. Rev., 21, 169-194.

Cole, K.S. (1932). Surface forces of the arbacia egg. J. cell. comp. Physiol. 1, 1-10.

Collander, R. & Bärlund, R. (1933). Permeabilitats-studien an Chara certatophylla. I. Die Permeabilitat für Nichterlectolyte. Acta. bot. fenn., 11, 1.

Collett, P. (1922) Couches minces de melanges binaires (glycerides). J. Phys. et Radium. 3, 128-132.

Crane, R.K. (1960). Intestinal absorption of sugars. Physiol Rev. 40, 789-825.

van Creveld, S. & Brinkman R. (1921). Ein direkter Bewiss fur die Impermeabilitat der Blut korperchea des Menschen und des kaninchens für Glucose. Biochem. Z., 119, 65-72.

Cruess, R.L. & Seguin F.W. (1965). Method described for application under nitrogen of samples of phospholipids to T.L.C. in order to prevent oxidation and to decrease the influence of atmospheric humidity on Rf values. J, Lipid Res., 6, 441.

Danielli, J.F. (1954). The present position in the field of facilitated diffusion and selective active transport. In "Recent Developments in Cell physiology". ed. Kitching, J.A., pp. 1-14. Butterworths Scientific Publications, London.

Danielli, J.F., (1958). Surface chemistry and cell membranes in "Surface phenomena in Chemistry and Biology". Editors, Danielli, J.F., Pankhurst, K.G.A. & Riddiford, A.C.

Davenport, J.B. (1964) Phospholipids of pigeon and ox skeletal muscle. Biochem J., 90, 116-122.

Davson, H. & Danielli, J.F. (1943). "The Permeability of Natural Mebranes". Cambridge Univ. Press,

Dawson, A.C. & Widdas, W.F. (1963). Inhibition of the glucose permeability of human erythrocytes by N-ethyl malemide. J. Physiol., 168, 644-659.

Dawson, A.C. & Widdas, W.F. (1964). Variations with temperature and pH of the parameters of glucose transfer across the erythrocyte membrane in the foetal fuinea-pig. J. Physiol., 172, 107-122.

Dawson, R.M.C. (editor) (1964). "Metobolism and Physiological Significance of Lipids". (Proceedings of Advanced Study Course held in Cambridge, 1963) Pub. London-Wiles.

Dawson, R.M.C. (1960). A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. Biochem J., 75, 45-53.

Dawson, R.M.C. (1965). Phosphatido-peptide like complexes formed by interaction of calcium triphosphainositide with protein. Biochem J. 97, 136-138.

Dawson, R.M.C., Hemington, N. & Lindsay, D.B. (1960). The phospholipids of erythrocyte 'ghosts' of various species. Biochem. J., 77, 226-230.

Day, T.D. (1960). Cited by Trevor I. Williams "The Elements of Chromatography". Blackie & Son Ltd.

Van Deenen, L.L.M. & de Gier J. (1964). Chapter 7., pp 243 - 287. Chemical composition and metabolism of lipids in red cells of various animal species, in "The Red Blood Cell". Eds. Charles Bishop & D.M. Surgenov. Acad. Press, New York - London.

Dikstein, S. & Selman F.G. (1965). Function of phospholipids in Receptors. Biochem. Pharmac., 14, 739-742.

Dixon, M. & Webb, E.C. (1958). Enzymes. Longmans, Green & Co.

Dourmashkin, R. Dougherty R.M. & Harris R.J.C. (1962). Electron microscopic observations on rous sarcoma virus and cell membranes. Nature, 194, 1,116.

Dodge, J.T., Mitchell, C. & Hanahan, D.J. (1963). Preparation and chemical characteristics of haemoglobin in free ghosts of human erythrocytes. Arch. Biochem. Biophys., 100, 119-130.

Dzieman, A.J. (1939). The lipid content and permeability of the erythrocyte. J. cell comp. Physiol, 14, 103-116.

Ege. R. (1920). Die Verteilung der Glucose zwichen Plasma und roten Blutkörperchen. Zur Physiologie des Blutzuckers. Biochem. Z. 111, 189-218.

Ege, R. (1921). Wie ist die Verteiling der Glucose zwichen den roten Blutkorperchen und der äusseren flussigkeit zu erklaren. Biochem.Z. 114, 88-110.

Ege, R., Gottlieb, N.W., & Rakestrow, N. (1925). Distribution of glucose between human blood plasma and red blood corpuscles and the rapidity of penetration. Am. J. Physiol, 72, 76.-83.

Ege, R. & Hansen, K.M. (1927). Distribution of sugar between plasma and red corpuscles in man. Acta. Med. Scand. Schwd., 65, 279-299.

Fahmy, A.R., Neiderwiser A, Patakig, & Brenner M, (1961). Dünnschicht- Chromatographie von Aminosauren auf kieselgel G.

Eine Schnellmethode zur Trennung und zum qualitativen Nachweis von 22 Aminosäuren. Helv. chim. Acta., 44, 2022.

Falta, W. & Richter-Quittner M. (1922) Nochmals zur Frage der Verteilung des Zuckers auf Blutkörperchen und Plasma. Biochem. Z., 129, 576-581.

Faust, R.G. (1960). Monosaccharide penetration into human red blood cells by altered diffusion mechanism. J. cell. comp. Physiol., 56, 103-121.

Ferandez-Moran, H. (1952). The submicroscopic oranization of vertebrate nerve Fibres. Expl. Cell Res., 3, 5-83.

Finean, J.B. (1954). The effects of osmium tetroxide fixation on the structre of myelin in sciatic nerve. Expl. Cell Res., 6, 283-292.

Finean, J.B. (1961a). The nature and stability of nerve myelin. Int. Rev. Cytol., 12, 303-336.

Finean, J.B. (1961b). The molecular structure of myelin. World Neurology, 2, 466-478.

Finean, J.B. (1964). The molecular structure of cell membrane. Brit. Biophys. Soc., Birmingham, April 1964.

Finean, J.B. & Robertson, J.D. (1958). Lipids and the structure of Br. Med. Bull. Vol. 14, no. 3, 267.

Finean, J.B. & Rumsby, M.G. (1963). Negatively stained lipoprtein membrane: The validity of the image. Nature, Lond., 197, 1326-1327.

Fischer, E.H. (1964). Binding of vitamin B6 coenzymes and labelling of active of enzymes by sodium boronhydride reduction. In "Structure and Activity of Enzymes." EditorsT.W. Goodwin, J.I. Harris & B.S. Hartley, Academic Press, London & New York.

Fischer, E.H., Kent, A.D., Snyder, E.R. & Kreiss, E.G. (1958). Reactions of sodium boronhydride with muscle phosphorylase. J. Am. chem. Soc.

80, 2906- 2907.

Fischer, M.H. & Moore G. (1907). On the swelling of fibrin. Am. J. Physiol., 20, 330-342.

Fisher, R.B. & Parsons D.S. (1953). Galactose absorption from the surviving small intestine of the rat. J. Physiol., 119, 224-232.

Fleischmann (1928). Untersuchungen zur Frage der Permeabilitat pflanzlicher und tierischer Zellmembranen fur Kohlehydrate. Plfügers Arc. ges. Physiol., 220, 448-465.

Formigne, N.J. & Pulie, J.A., (1957). Rodbard Clin. Chim Acta. 25. cited by Ansell & Hawthorne in "Phospholipids". Elsevier Publishing Co.

Forsling, M.L., Remfry, J.C. & Widdas, W.F. (1964). Uptake of dinitrofluorobenzene by human erythrocytes. J. Physiol., 17, 23-24p.

Forsling, M.L., & Widdas, W.F. (1964). Inhibition of the facilitated transfer of glucose in human erythrocytes by phenolphthalein. J. Physiol., 178, 12P.

Forsling, M.L. & Widdas, W.F. (1965). The effect of temperature on the parameters of glucose transfer in red cells and on those of competitive inhibitors. XXIII International Congress of Physiological Science - Abstracts of Papers - no. 130.

de Gier, J. & van Deenan, L.L.M. (1961). Some characteristics of red cell membranes of various animal species. Biochim. Biophys. Acta., 42, 286-296.

de Gier, J. & van Deenan, L.L.M. (1963). Specificity and variability of red cell lipids. Biochem J., 88, 39P-40P.

Glassman, B. (1926). Beitrage zur Physiologie des Blutzuckers. Hoppe-Seyler's Z. physiol. chem., 158, 113-138.

Goldacre, R.J.& Lorch. I.J(1950) Folding and unfolding of protein molocules in relation to cytoplasmic streaming, amoeboid movement and osmotic Work. Nature, 166, 497-500.

Goldrick, B. & Hirsch, J. (1964). A technique for quantitative recovery of lipids from chromatoplates. J. Lipid Res. 4, 482-483.

Gorter, E. & Grendel, F. (1925). On bimolecular layers of lipids of the chromocytes of the blood. J. exp. Med., 41, 439-443.

Green, D.E. & Perdue, J.F. (1966). Membranes as expressions of repeating units. Proc. natn. Acad. Sci. U.S.A., 55, 1245-1502.

Gryns, G. (1896). Über den Einfluss geloster stoffe aufdie rothen Blutzellen inverbindung mit den Erschunungen der osmose und Diffusion. Pflügers Arch. ges. Physiol., 163, 86-119.

Hadon, D.A. & Taylor J. (1963) The stability and properties of bimolecular leaflets in Aqueous solution. J. Theoret. Biol. 4, 281-296.

Hanahan, D.J., Dittmer, J.C. & Warashinn, E. (1957). A column chromatographic separation of classes of phospholipids. J. biol. Chem., 228, 685-700.

Hanahan, D.J., Watts, R. & Pappajohn D. (1960). Some chemical characteristics of the lipids of himan and bovine erythrocytes and plasma. J. Lipid Res., 1, 421-43.

Hanai, I, Haydon, D.A., & Taylor, J. (1964). An investigation by electrical methods of lecithin in hydrocarbon films in aqueous solutions. Proc. R. Soc A. 281, 377-391.

Hanai, I. & Haydon, D.A. (1966). The permeability to water of bimolecular lipid membranes. J. theor. Biol., 11, 370-382.

Hanai, T., Haydon, D.A., & Taylor, J. (1965). The influence of lipid composition and of some adsorbed proteins on the capacitance of black hydrocarbon membranes. J. theor. Biol., 9, 422-432.

Hand, D.W., Sandford, P.A. & Smyth, D.H. (1966). Polyphenolic compounds and intestinal transfer. Nature, 209, 618.

Harris, E.J. (1964). An analytical study of the kinetics of glucose movement in human erythrocytes. J. Physiol., 173, 344-363.

Harvey, E.N. (1931a). A determination of the tension at the surface of eggs of the Annelid chaetorperus. Biol. Bull. Woods Hole, 60, 67-71.

Harvey, E.N. (1931b). The tension of the surface of marine eggs especially those of the sea urchin Arbacia. Biol. Bull. Woods Hole, 61, 273-279.

Hedin, S.G. (1897). Über die Permeabilitat der Blutköperchen. Pflügers Arch. ges. Physiol. 68, 229-338.

Henderson, M.J. (1964). The uptake of glucose into cells and the role of insulin in glucose transport. Can. J. Biochem. Physiol. 42, 933-944.

Heinz, E. (1959). Exchange diffusion, transport and intracellular level of amino acids in Ehrlich carcinoma cells. J. biol. Chem., 233, 1488-1493.

Hillman, R.S., Landau, B.R. & Ashore, J. (1959). Structural specificity of hexose penetration of rabbit erythrocyte. Am. J. Physiol., 146, 1277-1281.

Höber, R. & Ørskov, S.L. (1933). Untersuchungen über die Permeirgeschwindigkeit von Anelectroylten bei der roten Blut körperchen verschiedener.

Tieraten. Pflügers Archs. ges. Physiol. 231, 599-615.

Hoeber, R. (1912a). Einzweites Verfahren die Leitfahigkeit im Inneren der Zellen zu messen. Pflügers. Arch ges. Physiol., 148, 189-221.

Hoeber, R. (1912b). Verteilung des Blutzuckers auf korperchen und Plasma. Biochem. Z. 45, 207-220.

Hoeber, R. (1910). Eine Methode die elektrische Leit fähigkeitim inneren von Zellen zu messen. Pflügers Arch. ges. Physiol., 133, 237-253.

Hokin, L.& Hokin, M.R. (1963). Biological transport. A. Rev. Biochem., 553-578.

Hokin, L. & Hokin, M. (1964). Incorporation of ^{32}P from triphosphate into polyphosphoinositide ($\lambda^{32}P$) adenosine and phosphatidic acid in erythrocyte membranes. Biochim. biophys. Acta., 84, 563-575.

Hokin, M.R. & Hokin, L.E. (1963). Interconversion of phosphatidyl inositol and phosphatidic acid in respect to acetylcholine in the salt gland. In "metabolism and Physiological Significance of Lipids - Ed. Dawson, R.M.C.

Hollinger, A. (1909). Uber die Verteilung des Zuckers im Blut. Biochem. Z. 17, 1 - 12.

Horne, R.W. (1964). Some recent applications of negative staining methods to the study of biological structure in the electron microscope. J. R. Microsc. Soc., 83, 169-177.

Horne, R.W. & Bangham, A.D. (1964). Negative staining of phospholipids and their structural modification by surface active agents as observed in the electron microscope. J. molec. Biol., 8, 660-668.

Huang, C & Thompson, T.E. (1965). Properties of lipid bilayer membranes separating two aqueous phases: Determination of membrane thickness. J. molec. Biol., 13, 183-193

Huang, C. Wheeldon, L & Thompson, T.E. (1964). The properties of lipid bilayer membranes separating two aqueous phases. Formation of a membrane of simple composition. J. molec, Biol., 8, 148-160.

Hughes,R.C., Jenkins, W.T. & Fischer, E.H. (1962) The binding of pyrixodal-5'-phosphate to heart glutamic-aspartic transaminase. Proc. natn. Acad. Sci. U.S.A. 48, 1615-1618.

Hunter, F.R. (1964). Permeability of erythrocytes to sugars III. A further analysis of the effect of tannic acid, mercury and triton-x-100. J. cell. comp. Physiol., 63, 39-54.

Hunter, F.R. George, J. & Ospina B. (1965). Possible carriers in erythrocytes. J. cell. comp. Physiol., 65, 299-312.

Izmailov, N.A. & Shraiber, M.S. (1935). Cited by T.R. Sas in "Apparatus for thin layer chromatography" Labatory Practice (1964). , 13, 311-314.

Kemmedy , E.P. Fox, C.F. & Carter, J.R. (1966) Membrane Structure and function. J. gen Physiol., 49, 347-354.

Kirschner, L.B. (1958). The cation content of phospholipids from swine erythrocytes. J. gen. Physiol., 42, 231-241.

Kleinzeller, A & Koty, A. (1961). Membrane Transport and Metabolism - Proceedings of a symposium held in Prague, August 1960. Academic Press.

Klinghoffer, K.A. (1935). Permeability of the red cell membrane to glucose. Am J. Physiol., 111, 231-242.

Koch, A.L. (1964). Role of permease in transport. Biochim. biophys. Acta, 79, 177-200.

Kotyk, A. (1964). Character of sugar carrier in different cells. Int. Biophys. Meeting, Paris.

Kotyk, A. & Höfer M. (1965). Uphill transport of sugars in the yeast rhodotorula gracilis. Biochim. biophys. Acta, 102, 410-422.

Kozawa, S. (1914). Beiträge zum arteigen Verhalten de roten Blut Körperchen. II. Kataphorese und Hämolyse. Biochem Z. 60, 146-158.

Kuhn, R., Winterstein, A. & Lederer, L. (1931). The xanthophylls Hoppe-Seyler's Z. physiol. chem., 197, 141-160.

Lacko, L & Burger, M. (1961). Common carrier system for sugar transport in human red cells. Nature, 191, 881-882.

Lacko, L. & Burger, M. (1962a). Interaction of some disaccharides with the carrier system for aldoses in erythrocytes. Biochem J., 83, 622-625.

Lacko, L. & Burger (1963). Kinetic comparison of exchange transport of sugars with non-exchange transport in human erythrocytes. J. biol. Chem., 238, 3478-81.

Lacko, L. & Burger M. (1964). The inhibition of transport of glucose into human erythrocyte by 2345 tetra (O-Acetyl) D glucopyranose. Biochem. biophys. Acta, 88, 564-569.

Laris, P.C., Novinger, G. & Calaprice, J. (1960). Phloretin, phloridzin and glucose inhibition of a phosphatase on the stromata of rabbit erythrocytes. J. Cell. comp. Physiol., 55, 127-129.

Lassen, U.V. (1961) Kinetics of uric acid transport in human erythrocytes. Biochim. biophys. Acta, 53, 557-569.

Lassen, U.V. (1962) Asymmetry of uric acid transport through the human erythrocyte membrane induced by hypoxanthine. Biochim. biophys. Acta. 57, 123-129.

Lasses, U.V. & Overgard-Hansen, K. (1962) Hypoxanthine as inhibitor of uric acid transport in human erythrocytes. Biochim. biophys. Acta, 57, 111-117.

Lassen, U.V., & Overgard-Hansen, K. (1962). Purine derivs as inhibitors of uric acid transport into human erythrocytes. Biochim. biophys. Acta, 57, 118-122.

Lea, C.H., Rhodes, D.N. & Stoll, R (1955). Phospholipids 3. On the chromatographic separation of glycerophospholipds. Biochem J. 60, 353-363.

Leathes, J.B. (1925). The role of fats in vital phenomena II. Lancet I 853-6.

LeFevre. P.G. (1948). Evidence of active transfer of certain non-electrolytes across the human red cell membrane. J. gen Physiol., 31, 505-527.

LeFevre, P.G. (1959). Molecular structural factors in competitive inhibition of sugar transport. Science 130, 104-105.

LeFevre, P.G. (1961a) Sugar transport in the red blood cell. Structure activity relationships in substrates and antagonists. Pharmac. Rev., 13, 39-70.

LeFevre, P.G. (1961b). Upperlimit for number of sugar transport sites in red cell surface. Fed. Proc., 20, 139.

LeFevre, P.G. (1961c). Persistence in erythrocyte ghosts of mediated sugar transport. Nature, 191, 970-972.

LeFevre, P.G. (1962). Rate and affinity in human red blood cell sugar transport. Am. J. Physiol., 203, 286-290.

LeFevre, P.G. (1966). The "dimeriser" hypothesis for sugar permeation through the red cell membrane. Biochim. biophys. Acta, 120, 395-405.

LeFevre, P.G. & Davies, R.I. (1951). Active transport into the human erythrocyte: evidence from comparative kinetics and competition among monosaccharides. J. gen. Physiol., 34, 515-524.

LeFevre, P.G., Habich, K.I., Hess, H.S. & Hudson, M. (1964). Phospholipid-sugar complexes in relation to cell membrane monosaccharide transport. Science, 143, 955-957.

LeFevre, P.G. & Marshall, J.K. (1958). Conformational specificity in a biological sugar transport system. Am. J. Physiol., 194, 333-337.

LeFevre, P.G. & Marshall, J.K. (1959). The attachment of phloretin and analogues to human erythrocytes in connection with inhibition of sugar transport. J. biol. Chem., 234., 3022-3026.

Levine, M. Oxender, D.L. & Stein, W.D., (1965). Substrate - Facilitated transport of the glucose carrier across the human erythrocyte membrane. Biochim. biophys. Acta, 109, 151-163.

Levine, R. & Goldstein, M.S. (1952). Brookhaven Symp., 5, 73.

Levin, R.J. & Smyth, D.H. (1963). The effect of the thyroid gland on intestinal absorption of hexoses. J. Physiol., 169, 755-769.

Liesgang, R.E. (1943). Cross capillary analysis, Naturwissenschaften, 31, 348.

Love, A.H.G., Mitchell, T.G. & Phillips, R.A. (1965). Cathartics and and the sodium pump. In Abstracts of papers presented at XIII Int. Congress Physiol. Sciences.

Lovelock, J.E., Webb, J.P.W., & James, A.T. (1959). The lipids of whole blood; I, lipid biosynthesis in human blood "in vitro". Biochem J. 73, 106-115.

Lovern, J.A. (1955). The Chemistry of Lipids of Biochemical Significance. Metheuns Monographs on Biochemical Subjects.

Lucy, J.A. & Glavert, A.M. (1964). Structure and assembly of of macromolecular lipid complexes composed of globular micelles. J. molec. Biol., 8, 727-748.

Luzzati, V. & Husson, F. (1962). The structure of the liquid crystalline phases of lipid water-systems. J. cell Biol., 12, 207-219.

Luzzati, V., Reiss-Husson, F. & Rivas, E. (1966) The structure and polymorphism in lipid water systems and their possible biological implications. Ann. N.Y. Acad. Sci., 137, 409-413. Monograph-biological membranes recent progress.

McKibben, J.M., & Taylor W.G. (1952). Isolation of a polyglycerol phosphatide from the alcohol insoluble phosphatide of dog liver. J. biol. Chem., 196, 427-436.

Maddy, A.H. (1964) The solubilisation of the protein of the ox-erythrocyte ghost. Biochim. biophys. Acta, 88, 448-449.

Mahadeven, V. (1962). Quoted by H.K. Mangold in "Thin Layer Chromatography". Ed. Stahl.

Mangold, H.K. (1964). Thin layer chromatography of lipids. J Am. Chem Soc., 41, 762-773.

Marinetti, G.V. (1962). Chromatographic separation, identification and analysis of phosphatides. J. Lipid. Res., 3, 1-20.

Martin, A.J.P. & Synge, R.L.M., (1941). A new form of chromatogram employing two liquid phases. 1. A theory of chromatography. 2. Application to the microdetermination of the higher amino acids in protein. Biochem J., 35, 1358-1368.

Martin, A.J.P. (1949). Chromatographic analysis. Discuss. Farady Soc. 7, 332-336.

Massing, E. (1912). Sind die Roten Blutkörpe durchgäning für Traubenzucker. Pflügers Arch ges. Physiol., 149, 227.

Matsumoto (1961). Biochem J. (Tokyo) 49, 11. Cited in "Phospholipds" B.B.A. Library, Vol. 3. editors Ansell, G.B. & Hawthorne, J.N. (1964). Elsevier Publishing Co.

Mawe, R.C. & Hempling, H.G. (1965). The exchange of ^{14}C glucose across the membrane of the human erythrocyte. J. cell. comp. Physiol., 66, 95-104.

Miller, D.M. (1965). The kinetics of selective biological transport II Equations for induced uphill transport of sugars in human erythrocytes. Biophys. J., 5, 417-423.

Miller, D.M. (1966). A re-examination of Steins dimer theory of sugar transport in human erythrocytes. Biochim. biophys. Acta, 120, 155-156.

Mitchell, C.D. & Hanahan, D.J. (1966). Solubilisation of certain proteins from the human erythrocyte. Biochemistry, N.Y., 5, 51.

Morell, S.A., Ayers, V.E., Greenwalt & Hoffman, P (1964). Thiols of the erythrocyte 1. Reaction of N-ethyl maleimide with intact erythrocytes. J. biol. Chem., 239, 2696-2705.

Morgan, H.E., Kalman, C.F., Post, R.L., & Park, C.R., (1955). Kinetics of glucose transport across the cell membrane. Fedn. Proc. 14, 103-104.

Morgan, T.E. & Hanahan, D.J. (1966). Solubilisation and characterisation of a lipoprotein from erythrocyte stroma. Biochemistry, N.Y., 5, 1050-1059.

Moseley, V & Chornock, F.W. (1947). Intubation studies of the human small intestine. XXV The absorption of galactose from the small intestine of normal individuals and thyrotoxic patients. J. clin. Invest., 26, 11-17.

Mueller, P. & Rudin D.O. (1963) Induced excitability in reconstituted cell membrane structure. J. theor. Biol., 4, 268-28

Mulder, E. & van Deenen, L.L.M. (1965). Metabolism of red cell lipids. III Pathways for phospholipids renewal. Biochim. biophys. Acta, 106, 348-356.

Mulder, E. Van Den Berg, H.W.O., van Deenen, L.L.M. (1965). Metabolism of red cell lipids. II Conversion of pysophosphoglycerides. Biochim. biophys. Acta. 106, 118-127.

Neuwirth, I. (1936). Distribution of glucose in blood. Am. J. Physiol., 117, 335-337.

Newey, H., Sanford, P.A. & Smyth, D.H. (1965). Uranyl ions and intestinal hexose transfer. Nature, 205, 389-390.

Nichols, B.W. (1964). The separation of Lipids by thin layer chromatography, Lab. Practice, 13, 299-305.

Olmsted, J.M.D. (1935). Distribution of Glucose in blood. Am. J. Physiol., 111, 551-553.

Ørskov, S.L. (1935). Eine Methode zur fortlanfenden photographischen Aufzeichnung von Volumanderungen der roten Blutkörperchen. Biochem. Z., 279, 241-249.

Osterhout, W.J.V. (1933). Kinetics of penetration. V. The kinetics of a model as related to the steady state. J. gen. Physiol., 16, 529-557.

Otto (1885). Virschow's Arch 35, 467. Cited by Pasquinell, F & Barsi, C. Sperimentale 1, 198-228.

Overton, E. (1899) Uber die allgemeinen osmotische Eigenschaften der Zelle, ihre vermuthlichen Vrasachen und ihre Bedeutung für die Physiologie. Verteljahrsh Naturforsch. ges. Zürich, 44, 88-135.

Park, C.R., Post, R.L., Kalman, C.F., Wright, H.J., Jr., Johnson, L.H. & Morgan, H.E. (1956). The transport of glucose and other sugars across cell membranes and the effect of insulin. Ciba Foundation Coll. Endocrin. 9, 240-260.

Park, C.R. (1961). In "Membrane Transport and Metabolism" (Proceedings of a Symposium held in Prague August 1963). Editors Kleinzeller. A. & Kotyk, A. Academic Press, London & New York.

Parpart, A.K., & Ballentine, R. (1952). Molecular anatomy of the red cell plasma membrane. In Modern Trends in Physiology and Biochemistry, p. 135-148 Ed. ESG Barron, Academic Press, N.Y.

Phillips, R.A., Love, A.G.H., Mitchell T.G. & Neptune, E.M. (1965). Cathartics and the sodium pump. Nature, 206, 1367-1368.

Ponz, F. (1945). La incluencia de al tiroxina sobre la absorcion intestinal de monosacarides. Reveta esp. Fisol, 1, 9-57.

Post, R.L., Merrit, C.R., Kinsolving, C.R. & Albright, C.D. (1960). Membrane Adenosine Triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. J. biol. Chem., 235, 1796-1802.

Quastel, J.H. (1965). Review Lecture - Molecular Transport at cell membranes. Proc. R. Soc. B., 163, 169-196.

Ramsay, Sir. Wm. (1894). On the passage of hydrogen through a a palladium septum and the pressure which it produces. Phil. Mag. 38, 206-218.

Randerath, K. (1963). Thin-layer Chromatography. Verlag Chemi & Academic Press.

Rathbone, L. (1962). Some observations on the chromatographic behaviour of phosphatidyl serine. Biochem J., 85, 461.466.

Reinwein, D. Kalman, C.F. Post, R.L & Park, C.R. (1955). Kinetics of glucose transport across cell membranes. Fedn. Proc. 14, 103-104.

Reiser, P. (1961). Sugar inhibited amino acid transport in the human erythrocyte. J. exp. Cell Res. 24, 165-167.

Reiser, P. (1962) The kinetics of valine transport in the human erythrocyte. J. exp. Cell Res. 27, 277-580.

Reiser, P & Reiser, C.H. (1964). Reversal of insulin resistance in resistance in red cell sugar transport. ArchsBiochem. Biophys. 105, 20.24.

Robertson, A.F. & Lands, W.E.M. (1964). Metabolism of phospholipids in normal and spherocytic human erythrocytes. J. Lipid Res., 5, 88-93.

Robertson, J.D. (1963). Unit Membranes - A review with recent new studies of experimental alterations and a new subunit structure in synaptic membranes. In "cellular Membranes in Development. Ed. M. Locke.

Rona, P. & Michaelis, L. (1909). Untersuchungen über den Blutzucker. VII Die Permeabilitat der Blutköperchen fuf Traubenzucker. Biochem Z., 18, 514-520.

Rose, H.G. & Oklander, M. (1965). Improved procedure for the extraction of lipids from human erythrocytes. J. Lipid Res., 6, 428-432.

Rosenberg, Th. & Wilbrandt. W. (1952). Enzymatic processes in cell membrane penetration. Int. Rev. Cytol., 1, 65-92.

Rosenberg, Th. & Wilbrandt W. (1957). Uphill transport induced by counterflow. J. gen. Physiol., 41, 289-296.

Rosenberg, T. Vestergaard-Bogind, B. & Wilbrandt, W. (1956). Carrier hypothesis of sugar transports. Helv. physiol. pharmac. Acta, 14, 334-341.

Rosenberg, Th. & Wilbrandt, W. (1952). Strukturabhangigkeit Hemmwirkung von Phlorizin und Adneren Phloretinderivation auf den Glukosetransport durch die Erythrocyten membran. Helv. physiol. pharmac. Acta, 15, 168-176.

Rouser, G., Kritchevsky, G., Heller, D & Lieber, E. (1963). Lipid composition of beef brain, beef liver and sea anemone. Two approaches to quantitative fractionation of complex lipid mixtures. J. Am. Oil Chem. Soc. 40, 425-454.

Rywosh, D. (1907). Verleichende Unter-suchungen uber die Resistenz der Erythrocyten einiger Saugethiere gegen hämolytische Agentien. Pflügers Arch. ges. Physiol., 116, 229.

Schmitt, F.O., Bear, R.S. & Ponder, E. (1936). Optical properties of the red cell membrane. J. cell. comp. Physiol. 9, 89-92.

Schmitt, F.O., Bear, R.S. & Ponder, E. (1938). The red considered as a Weiner mixed body. J.cell. comp. Physiol., 11, 309-315.

Schmitt, F.O. & Palmer, K.J. (1940). X-ray diffraction studies of lipide and lipide-protein systems. Cold Spring Harb. Symp. quant. Biol., 8, 24.

Schrier, S.L. (1966). Organisation of enzymes in human erythrocyte membranes. Am. J. Physiol., 210, 139-145.

Schulman, K.H. & Hughes, A.H. (1935). Monolayers of proteolytic enzymes and proteins. Biochem J., 29, 1243-1252.

Schwarz, H.P. (1960). "Infra-red absorption analysis of tissue constituents particularly tissue lipids. In "Advances in Clinical "Chemistry. Vol 3. pp. 1-34. Eds. Sobotka H and Stewart. Academic Press.

Sen, A.K. (1960). Factors affecting erythrocyte permeability to sugars. PhD Thesis, London.

Sen, A.K. & Widdas, W.F. (1960a). A new method for determining the half saturation constant of the transfer system for glucose in the human erythrocyte and for studying the effect of inhibitors. J. Physiol., 152, 32-33p.

Sen, A.K. & Widdas, W.F. (1960b). The effect of temperature and pH on the facilitated transfer of glucose across the human erythrocyte membrane. J. Physiol., 152, 64-65p.

Sen, A.K. & Widdas, W.F. (1962a). Determination of the temperature and pH dependence of glucose transfer across the human erythrocyte membrane measured by glucose exit. J. Physiol., 160, 392-403.

Sen, A.K. & Widdas, (1962b). Variations of the parameters of glucose transfer across the human erythrocyte in the presence of inhibitors of transfer. J. Physiol. 160, 414-416.

Shannon, J.A. (1939). Renal tubular excretion. Physiol Rev., 19, 63-93.

Skidmore, W.D. & Entenman, C. (1962). Two-dimensional thin-layer chromatography of rat liver phosphatides. J. Lipid Res., 3, 471-475.

Sjostrand, F.S. (1953). The lamellated structure of the mylein sheath as revealed by high resolution electron microscopy. Experientia, 9, 68-69.

Skipski, V.P., Peterson, R.F. & Barclay, M. (1962). Separation of phosphatidylethanolamine, phosphatidylserine and other phospholipids by thin-layer chromatography. J. Lipid Res., 3, 457-470.

Smith, I. (1960). Chromatographic and Electrophoretic Techniques. Vol. I. Chromatography. 2nd ed. Heinemann, London.

Solomon, A.K., Lionetti, F & Curran, P. (1956). Possible cation-carrier substances in blood. Nature, Lond., 178, 582-583.

Stahl, E. (1965). "Thin Layer Chromatography". A laboratory hand book. Berlin Springer.

Stein, W.D. (1958). N-terminal histidine at the active centre of a permeability mechanism. Nature, 181, 1662-1663.

Stein, W.D. (1961). Dimer formation and glucose transfer across the membrane of the red blood cell. Nature, Lond., 191, 1277-1280.

Stein, W.D. (1962). Spontaneous and enzyme induced dimer formation and its role in membrane permeability. III Mechanism of movement of glucose across the human erythrocyte membrane. Biochim. biophys. Acta 59, 66-77.

Stein, W.D. (1964). A procedure which labels the active centre of the glucose transport system of human erythrocytes. In "Structure and Activity of Enzymes" - Symposium no. 1. - Fed. European Biochem. Soc. Academic Press.

Steinbrecht, I. Hoffman E. (1962). Die Permeabilität von Kaninchenerythrocyten für 2-Desoxy-D-ribose. Hoppe-Seyler's Zeitschrift für Physiol. Chem. 329, 194-201.

Stoeckenius, W. (1962). Some electron microscopical observations on liquid-crystalline phases in lipid water systems. J. cell Biol., 12, 221-229.

Andreen-Svedberg, (1933). On distribution of sugar between plasma and corpuscles in animal and human blood. Skand. Arch. Physiol., 66, 113-190.

Tausky, H.H. & Shorr, E. (1953). A microcolorimetric method for the estimation of inorganig phosphorus. J. biol. Chem. 202, 675-685.

Teorell, T. (1952). Permeability properties of erythrocyte ghosts. J. Gen. Physiol., 35, 669-701.

Terroine, E.F. (1919). Ann. sci. nat. zool. 4, s40, 1. cited by Terroine (1935) in "Fat metabolism" Ann. Rev. Biochem., 5, 227-248.

Trappe, W. (1940). Separation of biological fats from natural mixtures by means of adsorption columns. Biochem. Z. 305, 150-161.

Tswett, M. (1903). Proc. Warsaw Soc. Nat. Sci. Biol., sect. 14, minute 6. cited by K. Randerath in"Thin-layer Chromatography". Verlag Chemie, Academic Press.

Turner, J.C. (1957). Absence of lecithin from red cell ghosts of ruminants. J. exp. Med., 105, 189.

Truter, E.V. (1963). "Thin Film Chromatography". Cleaver-Hume Press, London.

Ussing, H.H. (1947). Interpretation of the exchange of radiosodium in isolated muscle. Nature, 160. 262-263.

Ussing, H.H. (1952). Some aspects of the application of tracers in permeability studies. Adv. Enzymol., 13, 21-65.

Vogel, W.C., Doizaki, W.H. & Zieve, L. (1962). Rapid thin-layer chromatographic separation of phospholipids and neutral lipids of serum. J. Lipid Res., 3, 138-140.

Walden, (1898). Cited by W.M. Baylis in "General principles of Physiology", 3rd ed. (1917), Longmans.

Watkins, J.C. (1965). Pharmacological receptors and general permeability phenomena of cell membranes. J. theor. Biol., 9, 37-50.

Waugh, D.R., & Schmitt, F.O., (1940). Investigations of the thickness and ultrastructure of cellular membrane by the analytical leptoscope. Cold Spring Harb. Symp. quant. Biol., 8, 233-241.

Weed, R., Eber, J. & Rothstein, A. (1962). Interaction of Mercury with human erythrocytes. J. gen. Physiol., 45, 395-410.

Weed, R.I., van Stevinick, J. & Rothstein, A. (1964). Membrane sulphydryl groups and sugar transport in the red cell. Int. Biophys. Meeting, Paris, B. VI.

Weissman, G., Sessa, G. & Weissman, S. (1965). Effect of steroids "Triton X-100" on glucose filled phospholipid/cholesterol structures. Nature, 208, 649.

Whittam, R. (1964). Transport and diffusion in red blood cells- Monographs of the Physiological Society. Edward Arnold (Publishers) Ltd.

Widdas, W.F. (1952). Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. J. Physiol., 118, 23-39.

Widdas, W.F. (1953a). An apparatus for recording erythrocyte volume changes in permeability studies. J. Physiol., 120, 20-21P.

Widdas, W.F. (1953b). Kinetics of glucose transfer across the human erythrocyte membrane. J. Physiol., 120, 23p.

Widdas, W.F. (1954a). Comment on Professor Wilbrandt's & Dr. LeFevre's Papers. Symp. Soc. Exp. Biol. VIII, Active Transport & Secretion, 163-164.

Widdas, W.F. (1954b). Facilitated transfer of hexose across the human erythrocyte membrane. J. Physiol., 125, 163-180.

Widdas, W.F. (1963). Permeability. In "Recent Advances in Physiology" 8th edit. Ed. R. Creese & J.A. Churchill Ltd., London. pp. 1-35.

Wilbrandt, W. (1947). Die Wirkung des Phlorizins auf die Permeabilitat der menschlichen Erythrocyten für Glukose und Pentosen. Helv. physiol. pharmac. Acta., 5, 664-665.

Wilbrandt, W. (1950). Permeabilitätsprobleme. Arch. exp. Path. Pharm. 212, 5, 9-29.

Wilbrandt, W. (1951). Permeability active transport and carrier mechanisms. Dt. med. Waschr. 82, 1153-1158.

Wilbrandt, W. (1959). Permeability and transport systems in living cells. J. Pharm. Pharmac., 11, 65-79. Review Article.

Wilbrandt, W. (1963). Transport through biolological membranes. A. Rev. Physiol., 25, 601-630.

Wilbrandt, W., Gusenberg, E. & Lauener, H. (1947). Der Glucoseintritt durch die Erythrocytenmembran. Helv. Physiol. Acta, 5, C20-22.

Wilbrandt, W. & Kotyk, A. (1964). Transport of sugar mono and di-complexes in human erythrocytes. Naunyn-Schiedbergs Arch. exp. Path. Pharmak., 249, 279-287.

Wilbrandt, W. & Rosenberg, T. (1961). The concept of carrier transport and its corollaries in pharmacology. Pharmacol. Rev., 13, 109-183.

Williams, J.P.R. (1954). General Principles of chromatography. Br. med. Bull., 10, 165-169.

Williams, T.I. & Weil, H. (1952). A definition of chromatography. Nature, 170, 503.

Winkler (1938). Thesis Leiden. Cited Booij, - J.cell. comp. Physiol., 5, 495.

Winter, C.G. & Christensen, H.N. (1964). Migration of amino acids across membrane of human erythrocyte. J. biol, Chem., 239, 872-878.

Wolpers, von C. (1941). Zur seinstruckter der erythrocytenmembrane. Naturwissenschaften, 29, 416-420.

Additional reference

Stein, W.D. (1956). The permeability of erythrocyte ghosts. Expl. Cell Res., 11, 232-234.

Appendix i.

Correction to half-saturation for inhibitors.

The simplifying assumption is made that the inhibitor operates on the outside of the membrane and that the low glucose concentration is there in competition such that the transfer rate is modified by the factor $(1/1+x+y)$ where $x = \frac{C_g}{\phi_g}$ and $y = \frac{C_I}{\phi_I}$. C_g and C_I are the concentrations of glucose and inhibitor in the external solution and ϕ_g and ϕ_I are the half saturation constants for glucose and inhibitor.

When this factor is half the uninhibited value $(1/1+x)$ it follows that $y = 1 + x$ and hence

$$\frac{C_I}{\phi_I} = 1 + \frac{C_g}{\phi_g} \quad \text{and}$$

$$\phi_I = C_I \, 50\% \times \frac{\phi_g}{(\phi_g + C_g)}$$

Since ϕ_g varies with temperature the correction factor in brackets will also vary with temperature. In spite of the small value of C_g (0.7mM) the correction may be important when ϕ_g is also small. Thus using values of ϕ_g from Sen and Widdas (1962a) the corrections can be calculated at 37°C and 18°C as follows:-

at 37° $\quad \phi_g = 4 \quad \phi_I = \frac{1}{\left(1+\frac{0.7C_I}{4}\right)} = C_I \times 0.854$

and 18°C $\quad 1.31 \quad \phi_I = \frac{1}{\left(\frac{1+0.7}{1.31}\right)} C_I = C_I \times 0.655$

Appendix ii.

[*From the Proceedings of the Physiological Society*, 4 *June* 1964
Journal of Physiology, **173**, 23–24*P*]

Uptake of dinitrofluorobenzene by human erythrocytes

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It was shown by Bowyer & Widdas (1956) that 1-fluoro-2-4-dinitrobenzene (DNFB) was an irreversible inhibitor of facilitated glucose transfer in erythrocytes. When red cells were incubated with DNFB (1·4 mM) at 21° C, inhibition developed rapidly at first but took up to 4 hr to reach 99 % inhibition.

In the present experiments, the uptake of inhibitor has been measured using ^{14}C-labelled DNFB and parallel determinations of the inhibition of glucose transfer have been made. The uptake of radioactivity had a rapid initial phase followed by a more gradual rise, reaching about 2×10^9 molecules per erythrocyte when glucose transfer was 99 % inhibited.

By incubating with 1·4 mM DNFB at temperatures between 15° and 33° C, the time course for development of inhibition varied sixfold and the time course for uptake of radioactive DNFB was similarly affected. The relationship between inhibition and uptake was thus almost independent of the temperature of incubation. From the steepest part of the inhibition–uptake curve, it can be estimated that full inhibition would require a minimum uptake of 4×10^8 molecules per cell.

When butanol extracts of inhibited cells were fractionated it was found that labelling was present in some lipid fractions, but this represented less than 1 % of the DNFB taken up by the cells. That these lipids might ordinarily be capable of complexing with glucose is suggested by the observations of LeFevre, Habich, Hess & Hudson (1964) that the phospholipids of cells inhibited by DNFB are less able to promote the solubilization of glucose into hexane. The labelling appeared in fractions which were eluted from a silicic acid column by chloroform–methanol mixtures (9:1) and (1:4). The concentration of these compounds was of the order 4×10^6 molecules per cell and 3×10^6 molecules per cell respectively.

Stein (1964) has recently made a study of the peptides in erythrocyte ghosts which take up labelling when the erythrocytes are treated with radioactive DNFB at pH 6 and in 10 % alcohol. Peptide labelling in his experiments was of the order of 2×10^6 molecules per red cell ghost.

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REFERENCES

Bowyer, F. & Widdas, W. F. (1956). *Disc. Faraday Soc.* **21**, 251–258.
Lefevre, P. G., Habich, K. I., Hess, H. S. & Hudson, M. R. (1964). *Science*, **143**, 955–957.
Stein, W. D. (1964). *Abstr. 1st Meeting Fed. European Biochem. Soc.* D14.

Appendix ii.

[*From the Proceedings of the Physiological Society*, 16 January 1965]
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Inhibition of the facilitated transfer of glucose in human erythrocytes by phenolphthalein

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LeFevre (1959) showed that a number of synthetic oestrogens which had a diphenolic structure were potent inhibitors of glucose transfer across the erythrocyte membrane. As phenolphthalein has a diphenolic structure, its effect was tested by Dawson and Widdas (unpublished observations) who found glucose inhibition in foetal guinea-pig red cells. The inhibition has been further investigated on human erythrocytes, using the technique of Sen & Widdas (1962).

It has been confirmed that phenolphthalein is a competitive inhibitor of the facilitated transfer of glucose in human erythrocytes. Its potency lies intermediate between that of phloretin and phlorrhizin—a concentration of $20–25 \times 10^{-6}$ M produces 50 % inhibition at 37° C. There is no important change in inhibitory potency in the pH range 6–8·4.

The concentration which produces 50 % inhibition is reduced to 5×10^{-6} M if the temperature is lowered to 18° C. The Arrhenius plot is steeper than the corresponding plot for the half-saturation of glucose, suggesting that, with the inhibitor, there is a greater difference in the activation energies for complex formation and complex dissociation.

REFERENCES

LEFEVRE, P. G. (1959). *Science*, **130**, 104.
SEN, A. K. & WIDDAS, W. F. (1962). *J. Physiol.* **160**, 404.

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