EXTRACTION AND FRACTIONATION OF LIPID SAMPLES FROM RED CELLS FOLLOWING A BRIEF INCUBATION WITH ^{14}C-GLUCOSE

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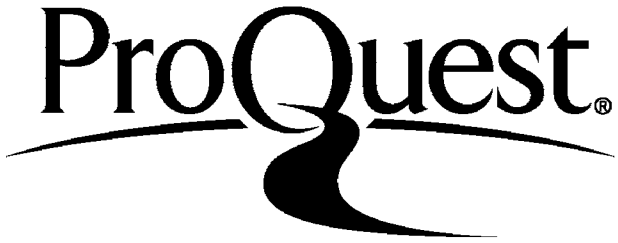

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A B S T R A C T

The facilitated diffusion of glucose across the human erythrocyte membrane has already been established by kinetic studies to be consistent with a carrier mechanism. Little is known biochemically about the carrier or the type of associations formed in its binding with glucose. In the experimental work to be described consideration was given to various possibilities of associations between membrane lipids and glucose molecules.

Human erythrocyte stroma were incubated with ^{14}C glucose for short periods at ice-cold temperatures and were then either extracted with lipid solvents, or freeze-dried prior to such extractions. Separation of the radioactivity and lipids in the extracts was effected by silicic acid/Hyflo column and thin-layer plate chromatography. Where possible a qualitative analysis was made of the lipid fractions associated with the radioactivity.

One of the kinetic features of the system in whole cells is the saturation of the carriers with increasing glucose concentration. Indications of this were looked for in experiments with increasing amounts of glucose but recoveries of radioactivity amongst the lipids did not appear to become saturated.

The effects of the reversible inhibitors phloretin and stilboestrol and of the irreversible inhibitors dinitrofluorobenzene(DNFB) and mercuric ions on the recovery of labelling and the chromatographic behaviour of the lipids and radioactivity have been studied.

Sheep erythrocytes have no detectable sugar transfer system and so a comparison was made between sheep and human erythrocyte lipid extracts following incubation of the cells with ^{14}C glucose under the same experimental conditions.

The main advances resulting from the experiments has been the demonstration of a lipid soluble ^{14}C glucose-containing material extractable from freeze-dried human ghosts by the methods used by Handa (1963) for glycolipids. The recovery of this material is markedly reduced by treatment of the ghosts with dinitrofluorobenzene or mercuric ions. On thin-layer chromatograms developed in dimethylformamide the 'complex' could be resolved from free glucose.

CHAPTER 1

Introduction

In 1895, Overton obtained the first evidence suggesting that exchanges between the cell and its environment are not always a case of simple diffusion. Using both plant and animal cells he showed that the cell allowed free passage of alcohols, aldehydes and alkaloids while the permeation of sugars and salts was hardly detectable. He concluded that the cell must therefore be bounded by an outer layer or membrane capable of selective permeability, by means of which it regulates its state and activity. The chemical properties of the permeates led Overton to suggest that the membrane is lipoid in character, consisting mainly of cholesterol and lecithin. Two years later, Hedin, working with ox erythrocytes, found that among non-electrolytes molecules of high lipid solubility penetrate most readily, the ease of penetration decreasing with increase of hydrophilic groups.

In 1925, Gorter and Grendel stimulated fresh interest in membrane structure by performing a simple technique for lipid investigation. Erythrocyte lipids from a known volume of blood were extracted with acetone and then applied to a Langmuir trough apparatus for determination of their total surface area. Assuming that the disc-shaped cells had a surface area of $2D^2$, where D is the diameter, the combined erythrocyte surface area was calculated. In each of the species studied it was found that the lipid area was twice that of the cell surface, suggesting that erythrocytes are covered by a bimolecular lipid layer.

In 1939, Dziemian expressed a similar view with the exception that he considered the lipid to be in the form of lipoprotein. In 1949, Ponder concluded from his immunological studies, measurements of electrical mobility and electron microscopy that the red cell would not be covered by a

continuous layer of lipid. Grendel and Gorter had considered the possibility that the non-polar groups of the lipids were orientated towards the outside and inside of the cell, with the polar groups held together in the middle possibly by calcium ions. But Danielli (1952) argued that such an arrangement of an outer layer of hydrophobic material would confer upon the cell a surface tension of 10-20 dynes/cm., whereas the measured value is of the order of 0.1 dynes/cm. Like Dziemian, he favoured a lipid-protein membrane where the lipid orientation was such that their polar groups faced towards the outside and inside of the membrane, and had a layer of protein absorbed on them. In that same year, 1952, Parpart and Ballentine proposed an arrangement of "lipid-protein" of red cell surface generally described as a mosaic arrangement. Cylinders of phospholipid and cholesterol of about 90 Å in diameter were arranged in a protein meshwork. The membrane could thus be described in terms of a mosaic in which a protein network of stromatin is interspersed with pores lined with lipid molecules.

Dourmashkin,* using negative staining techniques and subsequent electron microscopy, obtained micrographs of erythrocytes displaying a hexagonal array of pits 85 ± 5 Å in diameter. Each pit was surrounded by a ring 30 ± 5 Å thick which was hexagonal in outline. Prior treatment with digitonin prevented the formation of the pits, which seemed to suggest that the pits originally contained cholesterol. Sjostrand obtained similar micrographs with kidney tissue, mitochondrial membranes and smooth-surfaced cytomembranes. He has interpreted these observations in terms of an array of lipid molecules, in the form of globular micelles separated by protein molecules. The individual work of Glauert et al, (1964) and Bangham and Horne,+ has shown that artificial lipid mixtures resulted in lamellar, tubular, hexagonal and helical arrangements. Many of these structures could only be satisfactorily explained in terms of globular micelles of lipids and it was suggested

*Dourmashkin et al (1962) +Bangham and Horne(1962,1964)

that such may function as building blocks in the formation of biological structures containing lipids. But Glauert failed to find any regular geometrical structure associated with the erythrocyte membrane and suggested that the hexagonal structures observed by Dourmashkin could be artefacts due to rearrangement of the surface cholesterol in the absence of saponin during the preparation of the sections. The results suggest that there is no distinct individual repeating substructure in the plane of the erythrocyte membrane. Finean* made a comparison of the density diffraction patterns of epithelium, liver and kidney tissues with those of erythrocyte and myelin. From the results he deduced that in the rat erythrocyte density diffraction pattern, the pattern appears only along the equator, indicating an absence of sub-structures in the cell membrane. This evidence does not eliminate the ideas of the specific alignment of the lipid molecules within the erythrocyte membrane, as postulated by Lucy et al.(1964)

The predominant choice of the erythrocyte in all membrane studies unquestionably arises from the simplicity of its cellular structure. Histological staining has shown that the mature erythrocyte has lost both nucleus and mitochondria and while not possessing any of the specialised properties of other cells, e.g., secretion, electrical conduction, absorption, it does exhibit the common fundamental feature of regulating the entry and exit of substances between cyto-plasm and external medium. The membrane, as well as being chemically highly selective towards electrolytes such as sodium and potassium, exhibits similar selective permeability towards non-electrolytes and, in particular, towards the carbohydrates.

As early as 1914 Kozawa observed that rapid penetration of glucose was a property peculiar to primate erythrocytes, but that different monosaccharides permeated at different rates.

*Finean (1964)

From the discovery of Ørskov in 1935 that volume changes in the red cell could be followed photometrically originates most of the subsequent quantitative work on the rates of sugar penetration in the red cell. Confirmation of a number of early observed anomalies concerning glucose penetration was provided by LeFevre in 1948 using Ørskov's technique. Among such anomalies were the inhibition of penetration by heavy metals such as Hg^{++} by the chemical parachloromercuribenzoate and also by the drug phlorrhizin.

Similarly, in 1952, using the Ørskov-type apparatus, Widdas showed that glucose penetration was not a process of simple diffusion dependent on a concentration gradient. In a series of experiments where the concentration gradient was constant but the concentrations within the cells and medium increased, at the higher concentrations the penetration rate was greatly reduced.

In that same year LeFevre and LeFevre* sought to explain the kinetic anomalies on the basis of a carrier mechanism. This involved the combination of glucose with a membrane component effecting passage through the membrane. The model proposed assumed the complexes to form rapidly and the rate-limiting step to be the slow complex breakdown. Such a model provided an interpretation for their earlier observations of the variance of penetration rates of different sugars. They postulated that the sugars must therefore have different affinities for the transfer system, competing one with another for it. The aldohexoses had a high affinity for the carriers and showed marked inhibition to the penetration of ketose sugars such as sorbose and laevulose. Inversely, sorbose had no appreciable affect on glucose penetration. However, the model proposed did not satisfactorily explain the fall off in penetration rate at high concentrations observed by Wilbrandt, et al in 1947.

*LeFevre and LeFevre (1952)

In 1953, Widdas proposed a second model for glucose erythrocyte penetration which was an adaptation of a model he had earlier proposed for glucose penetration across sheep placental tissue. (Widdas 1951, 1952). In its simplest form it is assumed that the membrane components or carriers come into a rapid equilibrium with glucose in the medium at the inner and outer interfaces such that their saturation is determined by a relationship of the type

$$\theta = \frac{C}{C + \phi}$$

where C = glucose concentration and ϕ = half-saturation concentration. From this he deduced that the transfer rate for glucose (presumed to have a low value for ϕ) would be

$$\text{Transfer Rate} = \frac{K}{C_o C_i} (C_o - C_i)$$

where C_o and C_i refer to the outside and inside glucose concentration and K is a constant representing the maximal net transfer if one side is fully saturated.

Wilbrandt and Rosenberg* proposed a similar model to that of Widdas but with the inclusion of enzyme-catalysed steps in the combination between glucose and the carrier and also in the breakdown of such a sugar-carrier complex.

Definition of the system was given by Bowyer in 1957; it is such that the sugar combines with the carrier on the exterior cell membrane surface, the complex traverses the membrane to the interior cell surface where the complex dissociates, releasing the sugar into the interior of the cell. There is no requirement for metabolic energy and, although the penetration rate is not equivalent to a process of simple diffusion, the carriers and complexes can be regarded as diffusing down their respective concentration gradients. Such conditions of transport have become known

*Wilbrandt and Rosenberg (1951)

as facilitated diffusion.

In the bacteria similar transport systems exist for various metabolites, in particular the sugars and the amino acids, such that Monod* has categorised the types of transport into three main groups :-

(1) A specific transport process either thermodynamically active or passive. It involves the binding of the transported molecule onto a specific site present in the surface structure of the cell, and the ultimate result is the release of the unchanged transported molecule into the protoplasm as aqueous solute. Such a system is known as a permease system while the carrier itself is called the permease.

(2) An enzyme either at the outer surface of the membrane or excreted into the medium which breaks down the molecule for transportation, e.g., the hydrolysis of a disaccharide into two hexose molecules before the step of uptake. Such systems have been observed in yeast for disaccharides and for dextran sucrose and levan sucrose in the bacteria.

(3) Transfer where several enzymes are orientated in the cell such that the substrate binds to an active site on the external side of the membrane and the product of enzyme action is released into the intracellular fluid. In such a case the product is generally different from the original substrate.

It is group (1), the permeases, which are most like sugar transport in erythrocytes, with the exception that all recognised permeases perform active transport against the concentration gradient. Complete absence of passive transport is not, however, conclusive since, because of methodological reasons, it is difficult to clearly establish such a system. The transport systems of the bacteria exhibit similar properties to those of the erythrocyte-sugar

* Monod (1957)

transfer system showing saturation kinetics, competition of substrates, specificity of substrate and inhibition with certain chemical compounds and heavy metals.

The kinetics of both erythrocyte and bacterial transfer systems have been greatly elucidated by many workers over the last decade or so, but attempts at isolation of any particular membrane component involved in the tissues have so far proved relatively unsuccessful. In the case of bacteria the possibility of protein involvement is generally favoured, whereas with the erythrocyte there has been more investigation into the possible roles of the lipids within the membrane. Indeed, as early as 1902, Overton suggested that the sugar was transferred into a lipid-soluble compound, possibly by methylation. More recently Wilbrandt et al* investigated the possible transformation of the sugar into a lipid-soluble molecule. These workers synthesised such a compound in the natral ester glucose benzoate. They also showed that glucose-6-phosphate, glucose-1-phospate and hexose phosphate esters are incapable of penetrating the red cell.

In the erythrocyte stroma the respective weights of protein and lipid are approximately of the same order. The lipid content is composed chiefly of cholesterol and lecithin, but there is also present in varying amounts members of a group of lipids generally known as the phospholipids, of which lecithin itself is a member. In such lipids one of the hydroxy groups of the glycerol molecule is esterified with phosphoryl choline, phosphoryl ethanolamine or phosphoryl serine. When only one of the remaining hydroxy groups is esterified with a fatty acid moity, the resulting compounds are referred to as the lyso compound. Other lipids present are the phosphaditic acids, a group

*Wilbrandt et al (1956)

containing no bases and probably occurring in nature as hydrolysis products of choline, ethanolamine and serine phosphatides.

Other phospholipids containing no bases are phosphatidyl glycerol, cardiolipin and phosphoinositides. The sphingomyelins are composed of a polyvalent amino alcohol, phosphoryl choline and a fatty acid. Cerebrosides and gangliosides belong to a group called the glycolipids and contain sphingosine and a sugar. Into this group also fall a group of lipids recently isolated and identified by a group of workers in Japan, called the globosides. The major constituent of this group is globoside I which has the complex lipid structure of N-acetylgalactosaminoyl (1→3) galactosyl (1→4) galactosyl (1→4) glucosyl ceramide. A glycolipid of the same structure has also been isolated from human kidney by Rapport et al.(1964)

The percentage content of each phospholipid in red cells has been estimated by Hanahan* and by Dawson,+ and their individual results closely agree. Dawson also made a comparison of the phospholipid content of certain mammalian red cell stroma.

T A B L E I

PHOSPHOLIPIDS OF MAMMALIAN RED CELL STROMA

	Human	Pig	Horse	Cow	Sheep	Goat
Lecithin (+lysolecithin)	30.3%	25.1%	37.1%	14.1%	11.0%	12.0%
Choline plasmalogen	2.3	5.3	7.9	7.8	10.2	6.8
Sphingomyelin	19.3	15.4	23.8	31.2	37.0	35.8
Phosphatidylethanolamine	15.2	27.7	9.4	11.4	6.1	7.4
Total choline-containing phospholipids	51.9	45.8	68.8	53.1	58.2	54.6

*Hanahan (1960) + Dawson (1960)

It was found that cells with lower concentrations of lecithin contained more sphingomyelin such that the total choline-containing phospholipids remained fairly constant in the species examined. De Gier and Van Deenan (I96I) made a similar comparison on the erythrocyte stroma of sheep, ox, pig, man, rabbit and rat and also found that deficiencies in lecithin content appeared to be balanced by increased sphingomyelin, suggesting possible importance of the quaternary ammonium group to the cell surface. The relevance, if any, of phospholipid content in different species of animals to glucose transport is not evident. Apart from the slow glucose penetration observed in dog erythrocytes, rapid glucose penetration appears to be peculiar to primates. However, the foetal erythrocytes of many of those laboratory animals whose adult erythrocytes are apparently impermeable to glucose have a high rate of glucose penetration (Widdas, I955). This foetal erythrocyte transport system is in every way similar to that in adult human red cells.

The physical properties of the lipids at the membrane surface can be examined in '<u>artificial</u>' studies using the Langmuir trough apparatus. This technique provides a method for studying the arrangement of molecules in the peripheral region of the cell. The principles governing the orientation of substances at air-liquid interfaces can be applied to the interface between protoplasm and extracellular fluid. At a pure solvent-air interface the surface solvent molecules experience attraction by Van der Waals forces only at the side facing the solvent. Thus the molecules in the surface are in a state of tension, being at a higher free energy than those in the bulk of the solvent. However, with a solution, the introduction of the solute alters the intermolecular forces at the surface,

the result depending on the balance of interacting forces between solute and solvent. Certain molecules such as fatty acids, lipids, proteins and cholesterol are able to form a film on their solution surface. This requires (a) concentration of the solute at the surface and (b) the spreading out of the solute molecules to form a monomolecular layer on the surface.

The phospholipids are adequately suited to such requirements. The orientation of phospholipids in monomolecular layers depends not only on the nature of the paraffin chains but also on the phospholipid-sterol ratio. Van Deenan et al* investigated the reduction of the molecular area of certain phospholipid species by cholesterol. Using films of phospholipids, they recorded that the reduction depended largely on the nature of the fatty acids of the phospholipids. This indicates that, particularly in the myelin sheath, cholesterol may contribute to a highly organised alignment of the lipid constituents. Similarly, Gammack et al+ suggest dependence of ion selectivity in the neural membranes on the specific orientation and spacing of the lipid molecules. Certain acidic lipids including cerebral phospholipids and glycolipids have shown defined ion-exchange properties. Further studies on the properties of sphingomyelin monomolecular layers suggested they appeared to depend primarily on whether the predominant fatty acid was saturated or unsaturated. Replacement of a saturated by an unsaturated fatty acid resulted in the formation of a more expanded film.

The attachment of glucose to the lipid would alter the molecular area of the lipid and thus alteration in the surface area/pressure curves could be recorded. Experiments were carried out where a known quantity of glucose was added to the lipid water interface. Any alteration in the surface

*Van Deenan et al (1965) +Gammack et al (1965)

area/pressure curves was noted. In the present work various phospholipid monomolecular films were studied, among them cardiolipin, phosphatidylserine and triphosphoinositol. The results proved difficult in giving a clear analysis but suggested that the surface area/pressure curves of triphosphoinositide might be affected by the introduction of glucose to the medium.

With the recent improvement of techniques for the isolation and identification of the phospholipids, work in a number of laboratories has tended to focus on the isolation of phospholipid-sugar complex from Red Cellsystems. The erythrocyte stroma can be exposed to radioactive sugar for a short period (long enough for equilibrium to be established) and the membrane lipids subsequently extracted and examined for possible association with the radioactivity. Using such a technique, LeFevre* demonstrated the capacity of glucose to complex with certain of the phospholipids. However, similar results could be obtained by simple mixing of the glucose with the extracted lipids, thus pre-extraction lipid-sugar complex formation was not clearly established. Also the system did not exhibit any particular specificity since many of the other monosaccharides, e.g., D-mannose, D-ribose, D-lyxose, both enantiomorphs of xylose, galactose and arabinose, behaved similarly. In all cases the complex rapidly dissociated in the presence of water, the free radioactive sugar migrating to the water phase. Such migration was not a case of diffusion according to partition coefficients since the addition of lipid solution to a radioactive sugar solution did not result in any migration of radioactivity to the lipid phase. Thin-layer chromatography revealed that in the complexed sugar there was a predominance of lecithin associated with the radioactivity. LeFevre's work with artificial systems consisting of phospholipid solutions to which sugar was added showed that

*LeFevre (1964)

glucose was more soluble in highly non-polar solvents containing phospholipids as solutes than in the pure solvents.

The latter phenomenon was verified by using artificial systems which were analysed by thin-layer chromatography. Radioactive glucose plus certain individual phospholipids were incubated in a suitable lipid solvent. The post-incubation solutions were applied to thin-layer plates and radioactivity detected by means of controlled scanning. It was found that the amount of the glucose extracted by an organic solvent from a mixture of glucose and lipid was much greater than an extract of glucose alone, and this was so even in the complete absence of any subsequently detectable lipid complex. Equal volumes of each incubation mixture were applied. This apparent increased solubility of the glucose in the phospholipid solutions may be the attraction of the sugar to the hydrophilic groups of the phospholipid molecules and, in particular, to the phosphate groups.

Artificial phospholipid 'sacs' can be made by swelling the phospholipids in aqueous salt solutions to form crystals which behave as salt-containing compartments limited by discrete membranes. Weissman et al* prepared such cholesterol/ovolecithin spherullites and loaded them with glucose. The effect of steroids on leakage of glucose was then studied. All the steroids examined affected glucose leakage, but cortisol acetate, which appears to protect biological membranes, diminished the glucose release. The question was whether the steroids simply prevented glucose-lipid binding or did actually alter the spatial arrangement of the lipids. Electron micrographs of negatively-stained preparations showed that Triton X-100 effected complete destruction of the distinct spherullites, but there was an emergence of completely new forms. Some smaller spherullites

*Weissman et al (1965)

were observed but the predominant forms were a series of frayed, strand-like structures which had maintained a lamellar substructure. The results suggest that the steroid-induced changes in the permeability of glucose to phospholipid/cholesterol spherullites are the functional expression of a physical interaction between steroid and membrane lipid.

With the evidence from artificial systems confirming the possible formation of glucose-lipid complexes, it was decided that a tentative search for similar complexes should be made in the human erythrocyte membrane.

Red Cell Studies

Incubation of human erythrocytes in a sugar medium results in rapid equilibration of intra- and extracellular sugar. In the equilibrated state there is a constant exchange between the sugar of the cells and that of the medium. Carrier-bound sugar molecules are therefore constantly traversing the membrane effecting the exchange process. Extraction of the carrier membrane components would consequently result in the extraction of some sugar-carrier complex. Thus, were the carrier a lipid component, extraction of membrane lipids would result in the extraction ofa portion of complexed carrier. Use of radioactive sugar would enable the identification of the particular lipid component concerned. Investigations of this type were carried out to study those characteristics of erythrocyte-sugar transport previously observed in kinetic studies on the Ørskov-type apparatus.

(a) Extraction, isolation and identification of the lipids extracted from erythrocytes preincubated with ^{14}C glucose.

(b) The effect of increased sugar concentration in the incubation media on the lipid:sugar distribution.

(c) The effects of known inhibitors of the transport system on the lipid:sugar distribution.

(d) A comparison was made of results from sheep and human erythrocytes under similar experimental conditions.

In all the studies the erythrocytes were exposed to radioactive sugar for suitable periods of time at ice-cold temperatures. The membrane lipids were extracted with lipid solvents and separated by silicic acid column chromatography and thin-layer plate chromatography. The location of the radioactivity was determined by counting the fractions with a thin window Geiger Counter and by radioactivity scanning of the thin-layer chromatography plates.

Saturation of the Carriers

The degree of saturation of the membrane sugar carriers is determined by the sugar concentration of the medium in accordance with the Michaelis-Menten type kinetics where

$$\theta = \frac{C}{C + \emptyset} \qquad (I)$$

where θ = degree of saturation and C = sugar concentration of medium, $\emptyset$ is the half-saturation constant. At room temperatures the half-saturation sugar concentration value is of the order of 2mM, while at $0^{\circ}C$ it is less than I mM. (Sen & Widdas 1962) the lower temperature favouring complex formation. In experiment (b) an attempt was made to increase the sugar concentration of the incubation medium to give full saturation of the carriers, such that the quantity of sugar-complex material isolated would confirm a state of full saturation by reaching a maximum value unaffected by further increases in sugar medium concentration. The theoretical nature of saturation kinetics allow only an

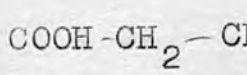
approximate calculation of sugar concentration necessary for full saturation of the carriers. A series of increasing sugar concentrations of the erythrocyte incubation medium were chosen for experiments in which attempts were made to obtain saturation of the carriers and to correlate the quantities of sugar associated with lipid fractions with the estimated degree of saturation.

Inhibition of Sugar Transport in the Human Erythrocyte

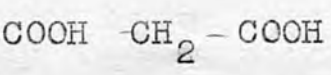
Kinetic studies have demonstrated the inhibition of glucose transfer in the human red cell by mercuric chloride, p-chloromercuribenzoate, phlorrhizin (LeFevre, 1948), stilboestrol (LeFevre,1959), phloretin (Wilbrandt 1950), polyphloretin phosphate (Wilbrandt & Rosenberg, 1950), 2:4 dinitro-fluorobenzene (DNFB), dinitrochlorobenzene and dinitrobromobenzene, urethane (Bowyer, 1954; Bowyer & Widdas, 1956), N-ethyl maleimide (Dawson & Widdas, 1963) and phenolphthalein (Forsling & Widdas, 1965). The distinctive modes of actions of the inhibitors can be compared with those of the enzyme substrate system in that they exhibit either competitive or non-competitive inhibition.

Competitive inhibition is often referred to as reversible inhibition and depends on the lack of absolute specificity of the chemical reactivity of the active site. The active site combines loosely with the inhibitor, being apparently incapable of distinguishing between substrate and inhibitor and able to combine with either. The degree of inhibition consequently depends on the substrate/inhibitor concentration ratio such that the inhibition effects can be reversed by increased concentration of substrate or decreased concentration of inhibitor. It is of interest that, in the enzyme substrate competitive inhibition systems, the substrate and inhibitor are often structurally related, e.g., succinic dehydroxygenase is inhibited by malonic acid:

Succinic Acid

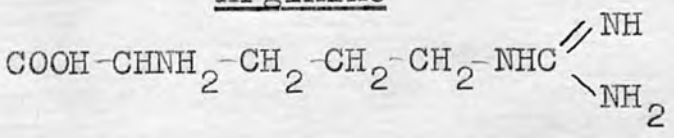


Malonic Acid



Similarly, arginase is inhibited by lysine:

Arginine



Lysine

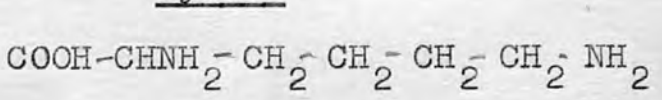


Fig. 1 shows the structure of four of the compounds known to reversibly inhibit sugar transfer in the erythrocyte. The fifth molecular structure is that of glucose. All four inhibitors possess two phenolic hydroxy groups having a centre-to-centre spacing of over 13 Å between the terminal hydroxyl oxygen atoms. LeFevre* has demonstrated the importance of such an arrangement to effective inhibition, proposing that the inhibition may be the result of reversible binding or association of the phenolic hydroxy groups with sites distributed in a recurrent spacing pattern over all, or part, of the red cell surface. Such sites are also the loci of the sugar transfer system.

The nature of the binding or association of sugar to complex is not one of chemical covalency but more of a type of loose association such as hydrogen bonding. Glucose is capable of hydrogen bond formation with a suitable acceptor site, e.g., the NH.OH group where there is an acidic hydrogen atom. Also all four inhibitors are capable of hydrogen bonding, e.g., phenolphthalein is known to exist in concentrated solution as a dimer resulting from intermolecular binding by means of hydrogen bond formation. In the dilute solution the dimer formation is broken by the hydrogen bonding between a phenolphthalein molecule and the more accessible water molecules. It is possible that when inhibitor and

* LeFevre (1959)

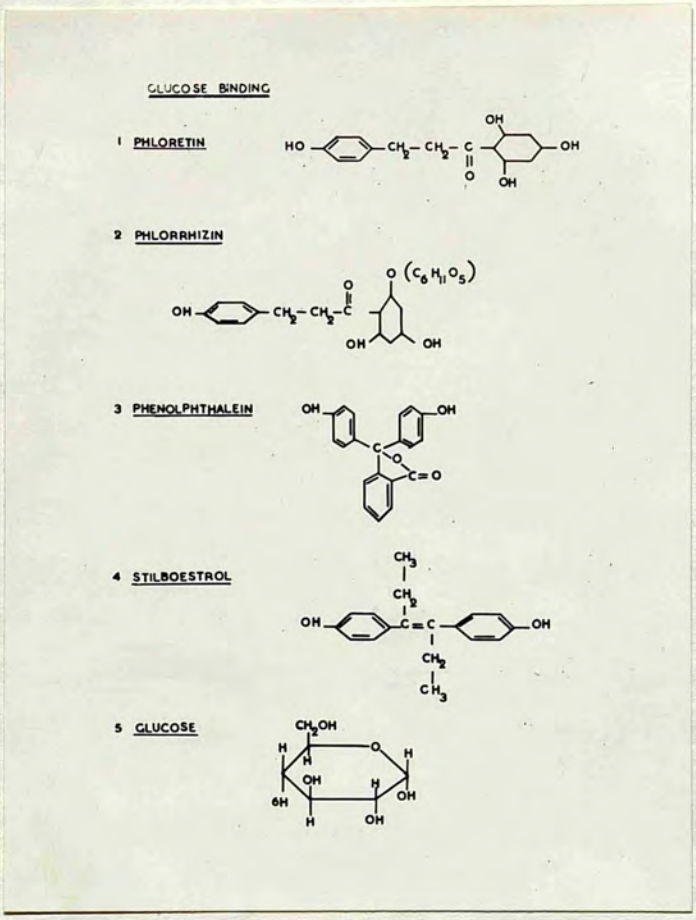


Fig. 1. The chemical structures of the reversible inhibitors:- 1. Phloretin, 2. Phlorrhizin, 3. Phenolphthalein, 4. Stilboestrol and 5. The structure of glucose.

glucose are present together, they would compete for any available hydrogen bonding sites such that the resulting equilibrium would depend on the relative concentrations of inhibitor and glucose. A possible kinetic test to such a postulation would be to study the inhibitive effects of a derivative of a reversible inhibitor in which the original free hydroxy groups were blocked by substitution of the hydrogen atom. A suitable derivative is that of stilboestrol where the two hydrogen atoms are substituted by propyl radicals.

Crane et al*, working with the sugar transfer system of intestinal tissue, found that the only essential structural feature for glucose transport was the hydroxyl group of carbon atom 2. They also found that iodoacetate inhibited the system, yet work with isotopes showed there was again no covalent bonds involved in the sugar-complex association. This system is an active one requiring expenditure of energy, the action of the iodoacetate may therefore have blocked an enzyme responsible for an exergonic reaction linked to the sugar transport. Alternatively, iodoacetate would attack the -SH groups of membrane proteins and lipids.

Non-competitive inhibition cannot be reversed by raising the substrate concentration. The inhibitors are believed to combine with the enzyme at a point other than the attachment of substrate but such that they are able to exert an effect on the active site from a distance. The substrate is then unable to prevent or to compete with the action of the inhibitor. In the erythrocyte-sugar transfer system the effects of non-competitive inhibition can sometimes be reversed by the washing of the cells, e.g., the inhibitor p-chloromercuribenzoate. The non-competitive inhibitors include SH-reagents such as p-chloromercuribenzoate, mercuric

* **Crane et al** (1963)

or gold chloride, 1-fluoro-2,4,-dinitrobenzene (DNFB) and N-ethyl maleimide. DNFB will react with free amino, tyrosyl or hystidyl groups with the formation of stable dinitrophenyl derivatives and the elimination of hydro-fluoric acid. It will also attack phenolic hydroxy groups and hydroxy groups, such as those of a hexahydric alcohol or even glucose itself under suitable conditions. These latter properties may prove more relative to the mechanism of inhibition by DNFB, particularly in the event of phospholipid involvement in sugar transport, since the latter are lacking in -SH groups but not in suitable hydroxy groups.

The heavy metals attack -SH groups and will also exhibit chelate bond formation at suitable sites.

Hunter (1964) demonstrated the irreversible inhibition of sugar transport in erythrocytes by the detergent Triton X-100. At concentrations above 0.5% haemolysis results. The mode of action of Triton X-100 on the membrane may be related to the observation of Weissman (1965) that this detergent disorganised the spatial orientation of lipids in artificial systems.

The addition of inhibitors to the erythrocyte sugar incubation mixture, and subsequent lipid extraction, would result in a decrease of radioactive sugar associated with any lipid component involved in sugar transport. The reversible action of the competitive inhibitors renders them less suitable to the extraction and isolation procedures, although experiments with stilboestrol and phloretin were performed. The irreversible action of the non-competitive inhibitors favours the experimental techniques performed in that the results obtained can be more easily related to the "pre-lipid extraction" state of the erythrocyte-sugar transport components. Experiments were performed with the non-competitive inhibitors N-ethyl maleimide, DNFB and mercurichloride.

Sugar Transfer in Human and Sheep Erythrocytes

Sheep red cells are among those of mammalian animals which have no detectable sugar transfer system. They were chosen because of their availability for a comparative study of results obtained from them and human erythrocytes under similar experimental conditions. At least three theoretical explanations can be postulated for the absence of the transport mechanism in the sheep erythrocytes :-

(i) There may be a complete absence of the membrane component(s) involved in sugar-complex association.

(ii) The components may be present in the membrane but in a form chemically or physically unsuitable for performing sugar transport. The inactivity would result from lack of specificity of the lipid alignment in the erythrocyte membrane or unsuitable spatial orientation of the lipid. Alternatively a chemical feature essential for carrier-sugar formation may be modified by, for example, substitution.

(iii) The third explanation is the absence of an enzyme essential for catalysis of any step of the sugar-complex reactions.

The study was made in an attempt to throw light on the chemical or physical features of the transport system essential to sugar transport which appear to be lacking in the sheep erythrocyte membrane.

Studies of Artificial Systems

These studies were performed in conjunction with the red cell work, and material from both systems was examined by similar techniques. The experimental procedure for the artificial systems consisted of the incubation of radioactive sugar with certain commercially-prepared phospholipids. After this incubation at room temperature, the resulting

system was redissolved in redistilled chloroform for application to silica gel G thin-layer plates. In all studies control systems of radioactive glucose alone were set up. The thin-layer plates were run in solvents selected to give separation of free from any bound glucose. The radioactive sugar was detected by radioactivity detection scanning. The solvent systems chosen were those recommended for lipid separation on thin-layer plates, but glucose is generally insoluble in such solvents and remains (unmoved) at the origin. In certain artificial and red cell fractions, the chloroform:methanol:water, 80:30:3, (by vol) system sometimes gave a suggestion of an adjacent peak of radioactivity or a trailing of the peak at the origin. A solvent system of dimethyl formamide gave a separation of the radioactivity into two fractions, one of R_f value 0.05, i.e., remaining at the origin, and the other of R_f value 0.80 - 0.85. A system of radioactive glucose alone was unmoved by dimethyl formamide, remaining at the origin with an R_f value of 0.05 - 0.07. Thus the earlier peak of radioactivity corresponds to free glucose. The artificial studies were performed in an attempt to identify the nature of material associated with the sugar of the second peak of R_f value 0.85.

Certain reversible and irreversible inhibitors were also added to the artificial lipid mixtures and the resulting radioactivity distribution recorded. Among those inhibitors used were phenolphthalein, which could later be detected by spraying the plate with alcoholic sodium hydroxide, mercuric ions and Triton X-100.

It was realised that the isolation of any lipid-complexed sugar did not necessarily confirm the involvement of that particular complex in the mechanism of sugar transport. However, such complexes could offer further information on the types of bonds and associations glucose is able to enter into which might be employed in the membrane of the erythrocyte and this information could be valuable in planning experiments to elucidate the nature of the facilitated transfer process.

CHAPTER 2

Experimental Procedures

Preparation of Red Cell Ghosts

Several pints of time-expired whole red cells of mixed blood groups were washed clear of free haemoglobin and cell debris. The washing solution was 0.9% saline, which was added to the cells, shaken and then centrifuged for 20 mins. at 3,000 r.p.m. The resulting supernatant and surface layer of white cell debris (buffy coat) was removed by a Pasteur pipette at a suction pump. The process was repeated until the supernatant was clear (usually after 3-4 washings). The whole red cells were then ready for incubation with glucose.

Few of the experiments were performed with whole red cells since the solvents used for lipid extractions invariably extracted haemoglobin also. The haem pigment proved a serious contaminant in later chromatographic and spectrophotometric work. It was therefore necessary to remove the haemoglobin from the whole red cells by preparing red cell stroma or ghosts.

Post et al (1950) developed a method for the preparation of red cell ghosts on a large scale. The cells were haemolysed by rapid immersion in distilled water and separated from the resulting haemoglobin solution by centrifugation in a high-speed centrifuge at low temperatures. The resulting red cell ghosts were washed until the supernatant was clear with tris/hydrochloric acid buffer, pH 7.4 and molarity 1×10^{-5}. This method was used to prepare the ghosts used in the experiments but with a higher molarity of tris/HCl buffer, viz. 1×10^{-4}, as suggested by Hokin & Hokin (1964). The higher molarity gave a greater reduction in haemoglobin content of the ghosts. EDTA was added to the buffer wash to

remove the heavy metals and all centrifugations were carried out at 2 - 4° C. Phase contrast microscope photographs of the ghosts confirmed that the rigid membrane structure remained intact, see Fig. 2. The photographs were similar to those obtained by Post et al. An initial volume of three pints of time-expired red cells yielded a 60 - 70 ml. volume of ghosts.

Preparation of the Radioactive Sugar Solutions

The radioactive sugar was supplied by The Radiochemical Centre, Amersham, in the form of a filter paper onto which the radioactive sugar was adsorbed. Slow elution of the filter paper with a 0.9% (w/v) solution of saline resulted in a saline solution containing the majority of the radioactivity. The solution was accurately made up with 0.9% saline to a recorded volume and comprised the stock ^{14}C-glucose solution. A standard ^{14}C-glucose solution was made by accurate volumetric dilution of the stock solution. Radioactive counting was carried out on samples of the standard ^{14}C-glucose solution, the counts providing a reference for all other calculations of sugar quantities. In the experiments in which increased sugar concentrations of the erythrocyte incubation medium were used, calculated quantities of analar ^{12}C glucose were added to the stock ^{14}C-glucose solution.

Extraction of Lipids from the Red Cell Membrane

Some lipids are bound to the protein units within the cell membrane so that the extracting solvent needs to break the binding links. Methanol, ethanol and acetone break a major portion of the linkages, but their relatively high polarity renders them unsuitable for solubilisation of the freed lipids. Thus the solvent systems most useful for the dual role of freeing and extracting the lipids are those where two or more solvents are used together in suitable volume ratios.

a.

b.

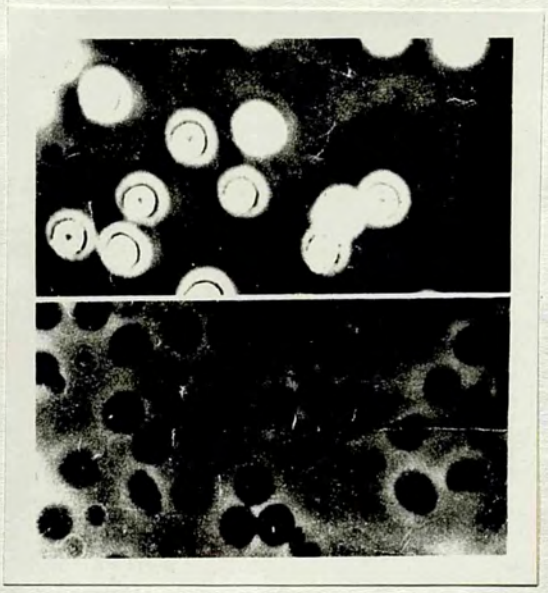


Fig. 2. Phase-contrast microscopy of :-

a. Intact whole red cells.

b. Red cell 'ghosts.'

The system with the widest application is probably that of Folch et al (1957) involving an extraction of the tissues with chloroform/methanol 2:1 (v/v). But this system is not altogether satisfactory for the extraction of the red cell lipids since Ways and Hanahan (1964) reported poor extraction of phospholipids and contamination of the extracts with water-soluble substances by this technique. An improved method was proposed by Reed et al (1960) using chloroform/methanol 1:1 (v/v) but involving four initial extractions and three further extractions of the residues. In addition, the initial extract was contaminated by denatured proteins and haem pigment.

Rose and Oklander proposed an improved procedure for lipid extraction from red cells using a simple solvent system of isopropanol and chloroform. A comparison of the results obtained with this solvent system and others is given in Table 1 (from Rose and Oklander, 1965), from which it can be seen that a solvent system of isopropanol/chloroform 11:7 (v/v) gives a higher yield of lipid extraction.

TABLE 1

Solvent Mixtures (by vol)	Total Cholesterol	Phospholipid
1. Chloroform-methanol 2:1	1.00 mg/ml cells	3.05 mg/ml cells
2. Chloroform-methanol 7:11	1.35 "	3.10 "
3. Chloroform-isopropanol 7:11	1.32 "	3.15 "
4. Chloroform-isobutanol 7:11	0.93 "	1.82 "
5. Chloroform-ethanol 7:11	1.35 "	3.27 "
6. Chloroform-butanol 7:11	1.20 "	3.00 "
7. Ether-ethanol 2:1	1.32 "	2.95 "

In solvent systems 2 and 5 there is also good extraction but these and some of the other systems suffered from pigment contamination, while the isopropanol/chloroform 11:7 (v/v) system gave no contamination of haem pigment.

Regardless of the extraction solvent system used, all lipid extracts will contain appreciable amounts of non-lipid contaminants. Among such are water-soluble contaminants which include: organic salts, amino acids, urea, sugars, nitrogenous bases, etc. Many of these water-soluble contaminants are the result of extractions from wet tissues and others are solubilised into the lipid solvents by the phospholipids. Folch et al removed much of the non-lipid contamination of a chloroform/methanol 2:1 (v/v) extract by mixing the lipid extract with 0.2 volumes of an 0.73% aqueous solution of sodium chloride. After centrifugation and separation of the two phases, the lower phase was repeatedly washed by the theoretical upper phase. The chloroform-rich lower phase contained the purified phospholipids. Dittmer et al (1957) achieved a high degree of phospholipid purification by a method involving re-extraction from ethanol-ether into petroleum spirit, followed by precipitation of the phospholipids with cold acetone.

The loose nature of the binding between sugar and hypothetical carrier molecules limited the methods of lipid extraction available. Any procedure involving a washing of the extract with highly polar solvents such as water or acid would inevitably favour the dissociation of the sugar-lipid complex. Among the solvent systems employed for the lipid extraction from red cells were butanol, chloroform-methanol 2:1, chloroform-ethyl acetate 2:1 and isopropanol-chloroform 11:7. The majority of extractions were performed with the latter solvent system. In earlier experiments a second extraction of the cells was performed using an acidified solution of the initial lipid extraction solvent. Certain of the proteolipids are only released in acidic medium and were thus extracted in the second solvent system. There were two major problems to this procedure which eventually terminated its use: (i) in acidic solutions glucose chemically

combines with aliphatic alcohols such as butanol, producing a compound which gave misleading results in the methods employed for lipid separation and identification; (ii) the acidic conditions increase the quantity of haemoglobin extracted, thus causing contamination of lipid fractions. A partial analysis of the results was possible.

Decreased contamination by water-soluble contaminants can be achieved by freeze-drying the cells before lipid extraction. Many of the later preparations were treated in this way. Thus the extraction procedures fall into two main groups: (a) the extraction of red cells or red cell ghosts with cold lipid solvents: (b) freeze-drying the red cell ghost preparations before extraction with cold lipid solvents.

Hot lipid solvents were unfavourable to the procedures since they encourage oxidation of, for example, unsaturated fatty acid moities.

The methods employed for separation of the lipids, e.g., silicic acid column chromatography and a series of preferential solvent extractions, also partially purify the phospholipids.

Separation and Analysis of Lipids

Although adsorption chromatography was discovered as early as 1903 by the Russian botanist Tswett, the real development of the technique did not begin until 1931. Tswett found that plant pigments could be separated by filtering a solution of them in petroleum ether through a column of calcium carbonate. Tswett chromatography was primarily suitable for the separation of lipophilic substances, and in 1931 Kuhn and Lederer introduced the method into the preparative chemistry of the polyene pigments. The rapid development of carotenoid chemistry in subsequent years was made possible by the use of chromatography on a preparative scale.

There was as yet no chromatographic method suitable for the separation of hydrophilic compounds such as proteins and nucleic acids. But in 1941 Martin and Synge introduced partition chromatography, from which arises most of the present-day techniques involving partition chromatography of both lypophilic and hydrophilic compounds. Their method consisted of columns of silica gel containing definite amounts of water and onto the top section of which the compounds for separation were adsorbed. A 'mobile' organic phase transported the compounds over the stationary 'aqueous' phase such that they were partitioned between the two phases. If the partition coefficients of the compounds to be separated differ sufficiently, they will travel different distances and are separated one from another in the column.

Later Consden, Gordon and Martin (1944) replaced the silica gel adsorption column by strips of filter paper, thus introducing the paper chromatographic technique. This method is more advantageous for micro-chromatographic separation but has the disadvantage of a long tedious preparation of suitable papers. Untreated papers give unsuccessful results, but filter papers such as Whatman's No.1, impregnated with silicic acid, are capable of separation of phospholipid mixtures. As yet the impregnated papers are not commerically produced and have to be made before use, a process detracting from their use. Nevertheless, paper chromatography, unlike column chromatography, can effect a complete separation of the lipids.

Silicic Acid Column Chromatography

The commercial production of a high grade of silicic acid has greatly enhanced its use in column chromatography. The successful separation by column chromatography of a mixture of substances is largely determined by two factors: (i) the reversible binding of individual components to the surface of the adsorbant, (ii) the ability of the eluting solvents to preferantially free them from the adsorbant. The adsorbants

are usually oxides such as silica which adsorbs the compounds onto its crystal lattice, binding them by electrostatic forces. Thus a more polar molecule is more firmly adsorbed and requires a highly polar solvent for elution. Thus a series of solvents of increasing polarity effects separation of the compounds according to their polarity. The neutral lipids and triglycerides can be eluted in the non-polar solvent, chloroform, while the phospholipids are eluted in a solvent of high methanol concentration.

In 1952 McKibbin and Taylor successfully separated a polyglycerol phospholipid from dog liver by gradient elution from a silicic acid column. Lea et al (1955) used the same technique to separate phosphatidylethanolamine and phosphatidylcholine from egg yolk. Hanahan et al (1957) extended the technique to the more complicated phospholipid mixture found in liver. With an increasing stepwise concentration of methanol in chloroform they separated all the main classes of phospholipids. Since then many workers have modified their basic technique for other separations.

Preparation of the Silicic Acid Columns

Working on the basis that 2-10 mg of dry lipid per gram of silicic acid gave good separation, suitable quantities of commercial silicic acid and Hyflo supercell were carefully weighed out. They were thoroughly washed in chloroform or chloroform-methanol 1:1 (depending on the first eluent), filtered on a Büchner funnel and finally dried in an oven for 12-16 hours at 110°C. The glass column used in all experiments had a diameter of 2 cm and a small disc of sintered glass at the bottom. The latter prevented the passage of silica into the eluent fractions. The activated silicic acid/Hyflo supercell mixture was suspended in approximately 100 ml of the first eluent, poured into the column and allowed to settle.

To pack the column firmly a further volume of first eluent was passed through and, at the latter stages, a small pressure from a nitrogen cylinder was applied. At no stage, either of packing or running of the column, was the eluent solvent allowed to reach the level of the silicic acid column surface. Any changes in eluent were performed at the point just before the level of the previous solvent coincided with the silicic acid surface.

The dried lipid extract was dissolved in redistilled chloroform and applied to the top of the silicic acid column. Elution was then performed starting with chloroform and continuing with a series of solvents increasing in methanol concentration. Nitrogen pressure was applied throughout the running of the column for two reasons: (i) to prevent oxidation of the lipids, (ii) to exert a pressure sufficient to give a suitable flow rate of approximately 1 ml/min. Individual fractions of 5 ml or 10 ml volumes were collected. Fractions collected from radioactive lipid extracts were estimated for radioactivity by drying aliquots onto planchettes and counting with a low background (thin mica window) Geiger Muller Counter.

Phosphate Estimations

As a guide to those fractions containing phospholipids, a phosphate determination was carried out on a sample of each of the fractions. The determination method chosen was a sensitive colourimetric one for samples of the order of 5 μg phosphorus. The method was developed by Taussky and Shorr (1953) and involves the use of ferrous sulphate in ammonium molybdate as the reducing agent producing a blue colouration. The method is preferable to the standard Fiske and Subbarow (1925) procedure on account of its sensitivity, ease of application and absence of any cloudiness in the developed colour. Using the spectrophotometer, accurate determinations of phosphorus per sample could be made. The quantities of fractions for

phosphate determination were evaporated to dryness and the phosphate hydrolysed with a solution of perchloric acid:10 N sulphuric acid 1:1 (v/v) for 18-24 hours, or until the hydrolysed solutions were clear. The hydrolysis was carried out on a sand bath at approximately 130°C in the fume cupboard. To each sample was then added a volume of reducing agent and, after shaking, the samples were left for 15 minutes for colour to develop before reading in the spectrophotometer at 690 mμ. From the results the amounts of inorganic phosphate present in the respective fractions were calculated.

Estimation of Carbohydrate and Sialic Acid

Carbohydrate estimations were made on the eluent fractions of silicic acid/hyflo columns. The method used was that of Yamakawa et al. (1960). The fractions were evaporated to dryness and the residue was dissolved in 0.5 ml. of distilled water. A solution of 100 mg. of anthrone and 2 gm. of thiourea dissolved in 200 ml. of 66% sulphuric acid was used for the colorimetric estimations. 5.0 ml. of this solution were added to the 0.5 ml. aqueous solution, which was then heated at 100°C for 15 minutes. In the presence of carbohydrate a green colour developed, and the optical density of the solution was recorded in the spectrophotometer at 615 mμ.

Sialic acid estimations were also performed according to the method of Yamakawa et al. To 1.0 ml. of aqueous solution 1.0 ml. of Bial's orcinol reagent was added and the resulting solution was heated at 100°C for 22 minutes. In the presence of sialic acid a greenish-blue colour developed, and the optical density of the solution was recorded in the spectrophotometer at 615 mμ.

Separation of lipid extracts on a silicic acid column is not always complete and there is overlap of eluted components but, when used in conjunction with the technique of thin-layer chromatography, a satisfactory separation is obtained.

Thin-Layer Chromatography

The limitations in the application of paper chromatography to lipophilic substances resulted in the development of a sensitive analytically-qualitative technique now known as thin-layer chromatography.

The technique was first described by Izmailov and Shraibner in 1938 when they dusted aluminium oxide onto glass plates and separated various compounds on these loose layers. Later Williams (1947) performed chromatography on layers of adsorbant between two glass plates, one of which had a small hole through which solutions and developing solvents were introduced. Meinhard and Hall (1949) were the first to employ a binding agent, such as starch, to give the layers greater mechanical stability. Kirchner et al (1951) developed the technique further and demonstrated its application to the separation and identification of terpenes using chromoplates on which several samples could be chromatographed. But the wide horizons of this field were not fully appreciated until 1956 when Stahl introduced a series of papers setting out a practical device for preparing chromatography plates consisting of layers of adsorbant 250 μ thick on glass plates. The adsorbant was called Kieselgel G and was silica gel with a plaster of Paris binding agent. He devised all the apparatus necessary for the preparation and spreading of adsorbant, and for the storing of the plates in between use. Most workers today use the Stahl devices or adaptations of them.

The thin-layer chromatography of the last ten years has demonstrated its advantages over paper chromatography for the separation of lipophilic substances. More recently suitable layers have been used for the separation of amino acids and nucleotides. The results were found to be equally as effective as, and in some respects superior to, paper chromatography.

The principles governing thin-layer chromatography have not been worked out in all their details since not all the factors are as yet clearly understood. But several authors, Stahl, Randerath and Smith, have set out most of the known factors affecting the technique.

The system can be classed as an 'open column' and basically consists of at least three components, viz., adsorbant, solvent and the compound being separated. The behaviour of the latter is dependent upon the adsorbant and solvent. The technique allows the prediction, within certain limits, of the suitability of a particular adsorbant/solvent system to the chromatography of a particular substance. The choice of adsorbants for a system is limited, while that of solvent is often varied such that the most suitable choice is largely determined by the experience of the individual worker.

Choice of Adsorbant

The adsorbants used in thin-layer chromatography are almost exclusively oxides, hydrated oxides or salts, probably the most widely used of all being the oxide silicic acid. The forces involved in the binding of compound to adsorbant are largely electrical surface forces which induce dipole moments in non-polar compounds and increase the existing dipole moments of polar compounds. Hydrogen bonds also often play a part in linking the adsorptive to the adsorbant. Among the adsorbants used are starch, calcium carbonate, silica, alumina acid and basic aluminium oxide, and activated charcoal. Of all adsorbants, silicic acid is the most widely used for the

chromatography of lipids. In the chromatography experiments performed the adsorbant used was silica gel G, the commercial product of Merck. Occasionally the chromatographed substance was investigated for sugar content and in such cases boric acid-impregnated silica gel G or sodium acetate-impregnated silica gel G was used. The apparatus and methods employed in the preparation of the thin-layer plates were those of Stahl. Five plates 20 cm x 20 cm were spread by mixing 25 gm of silica gel G, finely ground in a pestle and mortar, with 35.0 ml distilled water. A further 15.0 ml of distilled water was added while stirring, transferred to the spreader and the slurry was evenly spread over the plates. The whole process, after adding the final 15.0 ml of water, did not exceed 100 seconds. The spread plates were partially dried in air for 5 minutes and then finally dried and activated in an oven at 110°C for one hour. The activated plates were stored on a rack in a dessicator.

When boric acid-impregnated or sodium acetate-impregnated plates were prepared, the procedure was as above with the distilled water replaced by 0.1 M solution of boric acid or sodium acetate respectively. The plates were activated and stored as before.

Choice of Solvent Systems

The neutral lipids and triglycerides can be separated on thin-layer chromatography plates by relatively non-polar solvents, e.g., chloroform, diethyl ether, benzene:methanol 85:15, carbon tetrachloride and benzene; diethyl ether 50:50. But the separation of the polar phospholipids requires a relatively higher polarity of solvent system.

The lipid fractions for chromatography were often associated with radioactive sugar and the purpose of thin-layer separation was to isolate any lipid component in the mixture which retained the radioactivity. Glucose requires a relatively high polar solvent system for mobility on thin-layer plates, but the binding of lipid to sugar in the hypothetical

complex is such that in highly polar solvent systems such as water, acid or alkali, dissociation probably occurs. Chloroform:methanol:water systems have long been used for satisfactory separations of the phospholipids, but volume ratio of water required for glucose mobility is of an order favourable to complex dissociation. A solvent system of C:M:H_2O of 80:30:3 was used for many experiments, but the glucose remained at the origin. However, in a solvent system of C:M:H_2O of 60:35:8 the R.F. of glucose was 0.15 - 0.2 (see Fig. 4). But in the latter solvent system dissociation of any complex would be expected and possibly also in the former system. Nevertheless, lipids separated in such systems can be readily stained in a saturated iodine tank and thus serve to indicate those which may have previously been associated with the radioactive sugar.

In later experiments a solvent system of dimethyl formamide was used and was found to move certain fractions of the radioactivity. The solvent proved more suitable for the separation of radioactive lipid samples since it appeared to effect separation of the lipid components containing glucose from free glucose.

Some chromatography was performed on small plates 3¼" x 3¼". They were also spread with silica gel G and their use gives a greater speed of chromatography and is more suitable to the process of lipid detection by sulphuric acid charring. Various solvent systems comprised of different volume ratios of chloroform:methanol were used in an investigation into phospholipid separation in solvent systems without water.

Application of Samples to the Thin-Layer Plate

The application of samples is greatly simplified by the use of a piece of apparatus known as a template. The template is a perspex tray which, when inverted, fits completely over the plate and is graduated accurately for the application

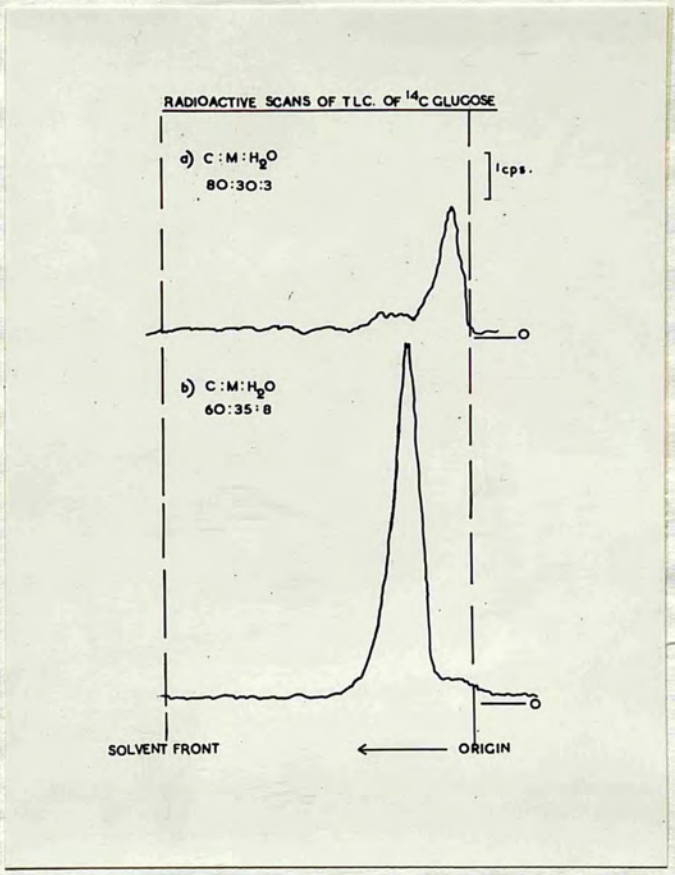


Fig. 4. Radioactive scans of ^{14}C glucose thin layer chromatographed in (a) C:M:H$_2$O, 80:30:3 (by vol) (b) C:M:H$_2$O, 60:35:8.

and running of the lipids. The maximum distance the solvent front was allowed to run was 10 cm. from the point of application of samples. In the case of the smaller plates the distance was 6.1 cm. All the samples were applied in redistilled chloroform using a Hamilton syringe which enabled the application of as little as 0.05 μl. The lipids were applied over a distance of 1 cm. in small successive quantities, each application being thoroughly dried before the next one. The volumes applied were calculated from the known or estimated concentrations of the samples, care being taken not to overload the plate either with lipid or radioactivity. A small hand-counter was used to detect the approximate quantity of radioactivity applied.

Preparation of the Chromatography Tanks

The tanks were made of thick glass enabling the observer to see the position of the solvent front on the plate for quick removal from the tank when the front reached the 10 cm. limit. The tanks were fitted with a glass lid which was coated with vaseline or suitable vacuum grease to ensure airtight sealing of the tank. The solvent system was poured into the tank to give a surface layer of 0.5 - 1.0 c.m. The tank was lined with Whatman's No.1 filter paper to ensure saturation of the tank atmosphere with solvent vapour. Rf values are strongly influenced by a saturated atmosphere since, in a multicomponent solvent system, the more volatile components evaporate more quickly, the rate of evaporation decreasing from the edge to the centre of the plate. Thus the Rf value would increase continuously from the centre to the edges of the plate. This effect can be avoided by uniform saturation of the tank atmosphere with solvent vapour.

The samples were applied to the plates, which were then placed into the saturated chromatography tank and only removed when the mobile solvent system had reached the 10 cm. limit. Before any staining techniques or radioactive detection

scannings were performed, the solvents were allowed to evaporate leaving the silica plate dry.

Staining Techniques Used

One of the simplest and most useful staining techniques for unsaturated lipids results from their reaction with iodine. The iodine reversibly saturates the double bonds, giving a brown or yellowish spot on the plate. Each phospholipid standard has a characteristic iodine spot which can be used for comparison with the unidentified samples. The main advantage of this technique is its reversibility. The plates were placed in a tank similar to the chromatographic tanks but with no filter paper and only sufficient iodine crystals to saturate the tank atmosphere. When the phospholipid standard spots had developed to a colour strong enough for examination or photography, the plates were removed. The iodine colour fades with time such that the plates can then be stained with a second reagent. Iodine colours are also given by saturated lipids containing nitrogen and esters of fatty acids. The plates were examined under ultraviolet light before any colour reagents were used.

Most spray reagents are liquids and it is essential for them to be sprayed uniformly onto the plate in a very fine dispersion. Non-corrosive spray reagents are commercially produced in spray tins while the corrosive reagents were carefully prepared and applied using a spray gun.

The phosphatide groups of the phospholipids can be readily stained to a blue colour with ammonium molybdate-perchloric acid solution. But this reagent lacks specificity, reacting with most lipids, and the colour development is slow at room temperature. A modification of this reagent was developed by Dittmer and Lester (1964) using the molybdenium blue reagent of Zinzadze. The mechanism for the colour reaction is still unknown.

The amino phospholipids such as phosphatidyl ethanolamine and phosphatidyl serine were detected using the Merck ninhydrin spray. The spray was uniformly applied to the plates which were then heated for 20 minutes at 110°C. The amino groups gave a red-violet colouration.

Acidic lipids were detected using the universal indicator Rhodamine-B (Merck). Red violet spots which fluoresced in ultraviolet light were produced, but there was a high background colour. When Rhodamine 6G is used, the spots are only visible on the wet plate under ultraviolet light. Thus the plate can be sprayed with a second reagent when dry.

In experiments where phenolphthalein was used as an inhibitor, the plates were sprayed with an alcoholic solution of sodium hydroxide. Under these conditions, the phenolphthalein gave the characteristic pink alkaline colouration.

Any organic material will char when sprayed with 20% sulphuric acid and strongly heated on an electric hot plate. Such treatment would risk breakage of 20 cm x 20 cm plates so the small thinner plates were used for this purpose. The silica suspension spread onto the smaller plates was calculated to give the same density as that of the larger plates. The solvent running distance was 6.0 - 6.2 cm., the spots being applied 1 cm. from the plate edge. Several solvent systems were used for development, some being more suitable than others. The plates were viewed under ultraviolet light and scanned before spraying with 20% sulphuric acid and charring. Several colour changes were observed as the heat intensity increased during charring, e.g., cholesterol and cholesterol esters were first red, then brown, then black; derivatives of vitamin A and its esters were first blue, then black.

Aniline Phthalate (Merck) was used for sugar detection, although the quantities of sugar on the plates were usually too low for detection. The plates were sprayed, then heated at 110°C for 10 minutes. The aldopentoses were reddish-brown in colour, while the methyl pentoses and aldohexoses were brown in colour.

Infra-Red Spectrophotometer

Carbon tetrachloride solutions and paraffin mull preparations were made of some samples for the recording of their infra-red spectra in the Perkin-Elmer spectrophotometer. Low quantities of material and impure standards, however, prevented accurate interpretation of the results.

Sephadex Columns

Dextran chains will swell in solutions to give large porous chains of molecules which, when packed into a vertical column, act as a molecular sieve on any mixture of compounds applied to the top of the column. The smaller molecules enter the molecular pores and require the flowing of solvent to wash them out. The larger molecules cannot enter the pores and pass through the column undetained. The smaller the molecule the more likely it is to fall into the molecular pores and the longer it will remain in the dextran column. This process of separation according to molecular size is known as gel filtration. The forces involved depend largely on their molecular size and not on the polar nature of the individual components. All components of an applied mixture are washed off the column in 2-2½ times the volume of the column bed. The dextran material, or Sephadex, is easily washed for re-use.

Until recently the commercial grades of Sephadex available were only capable of swelling in watery or very polar solvents. Now Sephadex G 15 and LH-20 are capable of swelling in organic solvents such as methanol, ethanol, dimethyl formamide and ethyl acetate. Sephadex LH-20 is Sephadex 20 in which the dextrans have been methylated. The products G 10 and G 15

were used with a dimethyl formamide solvent system in an attempt to effect the separation of free from lipid-bound glucose. The latter would be eluted first from the column. Several length columns were used the largest being 100 cm. long and fitted with a teflon tap. The Sephadex was suspended in dimethyl formamide until swollen and then packed into the column at the bottom of which was a silica stop covered with a layer of glass beads. When sufficiently packed the material for separation was applied in 1 ml. of dimethyl formamide and fractions of 2.5 ml. were collected. Since the sugar was radioactive, counts were taken of each sample. This work is in its very early stages and the behaviouf of the phospholipids under such separation techniques is not yet known. But it did serve to indicate the presence of any larger sugar-lipid moities in a radioactive sample.

The Preparation of Triphosphoinositide

Dittmer and Dawson in 1961 isolated a new group of phospholipids from brain called the phosphoinositides. There are three such phospholipids, mono-, di- and triphosphoinositide and Hokin and Hokin (1964) showed that the monoform was rapidly converted to the tri-form when P^{32} phosphate was introduced into the tissue. The amounts of each present in red cells is very small, while in brain all three are present in larger amounts. Brain tissue was therefore used for the isolation of a sample of triphosphoinositide according to the method of Eichberg and Dawson (1964). Four rats were decapitated and the heads immersed immediately into a dricold-acetone solution for rapid fixation of brain tissue. This procedure prevents post-mortem breakdown of triphosphoinositide. The brain tissue was removed, cleaned of blood clots, then homogenised with five times its volume of chloroform-methanol 1:1 (v/v) with an M.S.E. homogeniser. Most of the cholesterol, triglycerides, neutral lipids and some of the phospholipids,

were removed in this extraction. The solution was filtered through scintered glass and a second extraction with chloroform-methanol-conc. HCl 200:100:0.25 (v/v/v) was performed on the tissue residue. The tissue was extracted twice with this solution for 20 minutes at 37°C. The collective volumes of extract were centrifuged and the resulting supernatant removed. The triphosphoinositide was released from any bound protein by washing the extract with normal hydrochloric acid. The free triphosphoinositide passed into the chloroform-rich lower layer which was separated from the upper layer and washed several times with the theoretical upper layer of hydrochloric acid-methanol-chloroform 47:48:3 (v/v/v). The resulting solution was evaporated to dryness under reduced pressure in a nitrogen atmosphere and the yield of triphosphoinositide estimated. Thin-layer chromatography showed the sample to contain at least five components but was relatively pure and not greatly inferior to a 90% pure sample contributed by Dr. Dawson. A commercial preparation of diphosphoinositide is now available from Koch Light & Co.

The Isolation of Globosides from Red Cells

Handa and coworkers (1963) developed a series of solvent extractions on red cell stroma which resulted in the separation of a group of glycolipids called the globosides. The structure of globoside I was shown to be N-acetyl-galactosaminoyl:(1→3) galactosyl (1→4) galactosyl (1→4) glucosyl ceramide. The extraction procedure was a series of solvent extractions as shown in Fig. 5. The same series of solvents as those for isolation of methanol-ether globosides was used on freeze-dried ghosts which had been incubated with ^{14}C-glucose. The quantity and character of the radioactivity in each solvent was examined. The technique resulted in partial separation of free radioactive sugar from bound radioactive sugar.

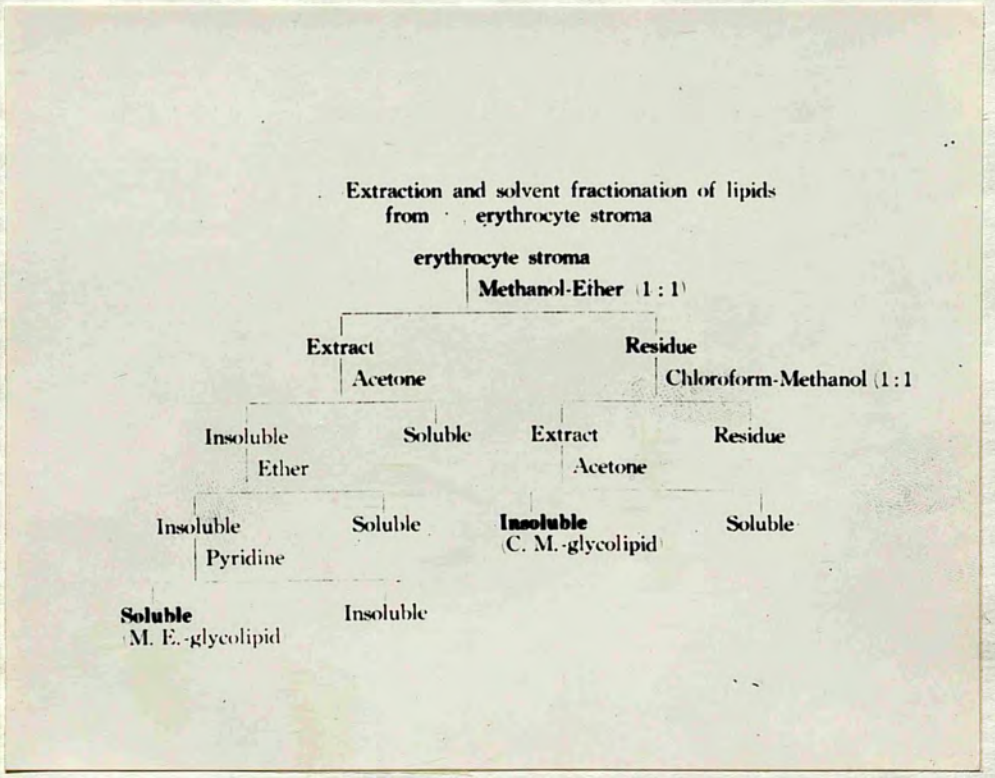


Fig. 5. The extraction procedures for the isolation of methanol:ether glycolipids as described by Handa et al (1963).

Artificial Systems

Radioactive sugar was incubated with lipid samples in pyridine as solvent, either for one hour at room temperature or for longer periods in the refrigerator. Pyridine was found to be the most suitable incubation medium since the solubilities of both sugar and lipids in this solvent were adequate for the possibility of complex formation. The behaviour of the inhibitors phenolphthalein, mercuric chloride and Triton X-100 in such artificial systems was also recorded. After the selected period of incubation, the pyridine was evaporated under nitrogen and the samples were applied in redistilled chloroform to thin-layer plates for subsequent detection and scanning.

CHAPTER 3

Experimental Data and Results

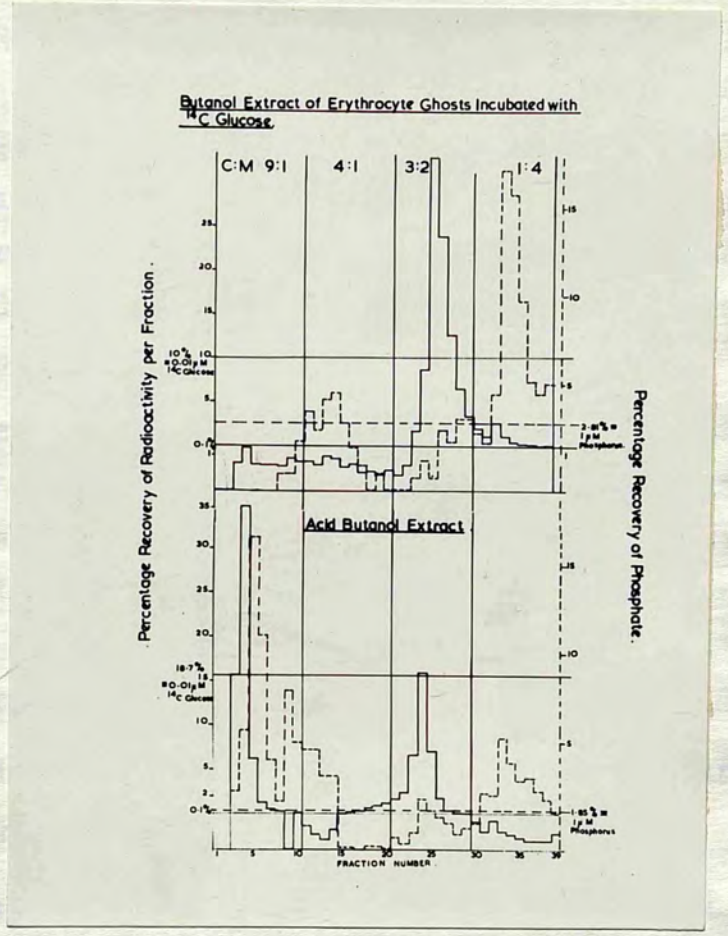
a. Red Cell Ghosts incubated with ^{14}C Glucose

60.0 ml. of human erythrocyte stroma were incubated with 9.0 ml. of a stock ^{14}C glucose solution and extracted with cold butanol under the described experimental conditions. The total activity of the added glucose was estimated at 4.44 million counts per minute and 2.5 million counts per minute of this were recovered in the butanol extract. A second extraction was performed on the residue with an acidified butanol solvent (100:0.25 v/v, butanol:conc.HcL). This extract yielded a further 0.8 million counts per minute. Both extracts were evaporated individually to dryness in an atmosphere of nitrogen under reduced pressure and the residues were redissolved for silicic acid/Hyflo column chromatography. The same series of eluents was used in each column and radioactivity and phosphate were measured on each fraction. The results, in terms of percentage recovery of total radioactivity and percentage recovery of phosphate, are represented on Graph 1.

The radioactivity of the butanol extract was resolved into four main regions of radioactivity :-

	Tube No.	Eluent	Counts/Min.	^{14}C Glucose
I	3 - 4	C:M 9:1	2,000	159 pmoles
II	13 - 14	C:M 4:1	1,900	150 pmoles
III	23 - 30	C:M 3:2	1,207,000	0,096 μmoles
IV	32 - 34	C:M 1:4	59,000	0.0048 μmoles

Regions 1, 2 and 3 indicated on the graph were selected for silica gel G thin-layer plate chromatography in a solvent system of chloroform-methanol-water 80:30:3 (v/v). After separation and drying of solvent, the plate was developed in



Graph 1. Silicic acid/Hyflo columns of butanol and acid:butanol extracts. The percentage recovery of radioactivity and the percentage recovery of phosphate per fraction are represented by the continuous and broken line graphs respectively.

a saturated iodine tank (Fig.6). It was seen that all samples were heterogeneous and no one lipid appeared to be in a predominant concentration. Radioactivity scanning showed that Region I had one peak of radioactivity attributable to free glucose of R.F. 0.05. Region II had two peaks of radioactivity, but Region III only had one radioactive peak corresponding to that of free glucose. This peak agreed with the silicic acid chromatography of a sample of ^{14}C glucose (Graph 2) where the radioactivity appeared only in Region III.

All three samples were eluted from the plate and ^{12}C-glucose added to each to observe the effect on the scanning behaviour of the rechromatographed material. In Sample I the peak was smaller and a little earlier. In Sample II both peaks were present but a little smaller, while in Sample III the peak was unreduced.

The radioactivity of the acid/butanol extract was resolved into two main regions :-

Tube No.	Eluent	Counts	Moles Glucose
3 - 6	9:1	466,500	0.037 μmoles
23 - 25	3:2	237,350	0.018 μmoles

All tubes 3 - 6 were contaminated with haem pigment, tube 3 having the strongest colouration, which caused severe streaking when chromatographed on the thin-layer plates. Subsequent iodine staining only enhanced the streaking effect (Fig.7) so that the resulting plate was impossible to interpret.

Radioactivity scanning showed Region I had two peaks, one attributable to free glucose and the other a prolonged peak probably associated with the streaking of the Haem pigment on the plate. Region II had only one peak of the same Rf value as free glucose.

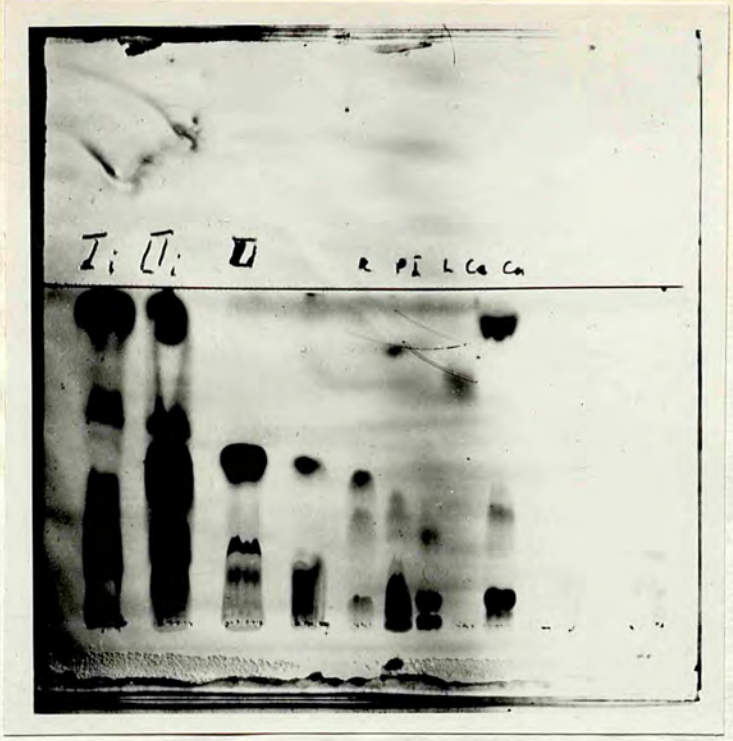


Fig. 6. Thin layer chromatogram of regions I (Fractions 3 and 4), II (Fractions 13 and 14) and III (Fractions 23-30) of the silicic acid column of the butanol extract. The plate was chromatographed in C:M:H_2O, 80:30:3 and developed in a saturated iodine tank.

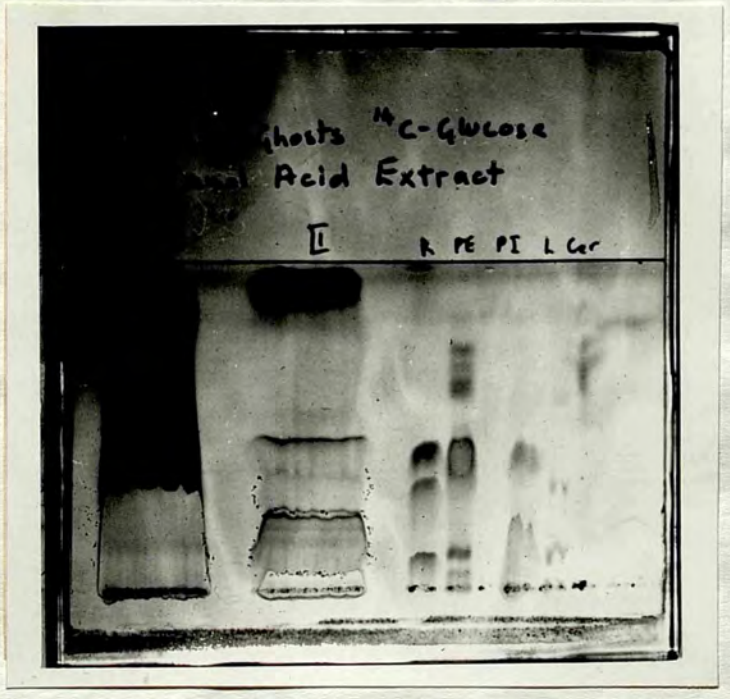
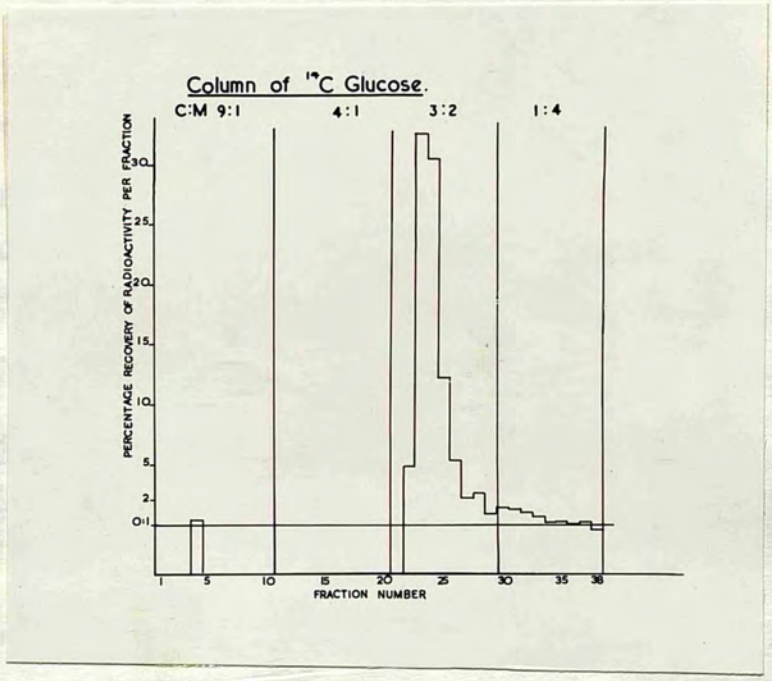


Fig. 7. Thin layer chromatogram of regions I (Fractions 3 and 4) and II (Fractions 23-25) of the silicic acid column of the acid:butanol extract. The plate was chromatographed in C:M:H_2O,80:30:3 and developed in a saturated iodine tank.



Graph 2. Silicic acid/Hyflo column elution of ^{14}C glucose.

(v/v) Previous workers had found that chloroform-methanol 2:1 did not extract a very large proportion of radioactivity from red cells incubated with ^{14}C glucose, but the resulting extract had a much shorter period of evaporation to dryness for column chromatography than the period of evaporation of butanol. The large non-free glucose peak of the radioactive scan of Region I of the acid/butanol column was looked for in a similar experiment where acid-chloroform-methanol (0.25:200:100 v/v) was used as the second lipid extracting solvent.

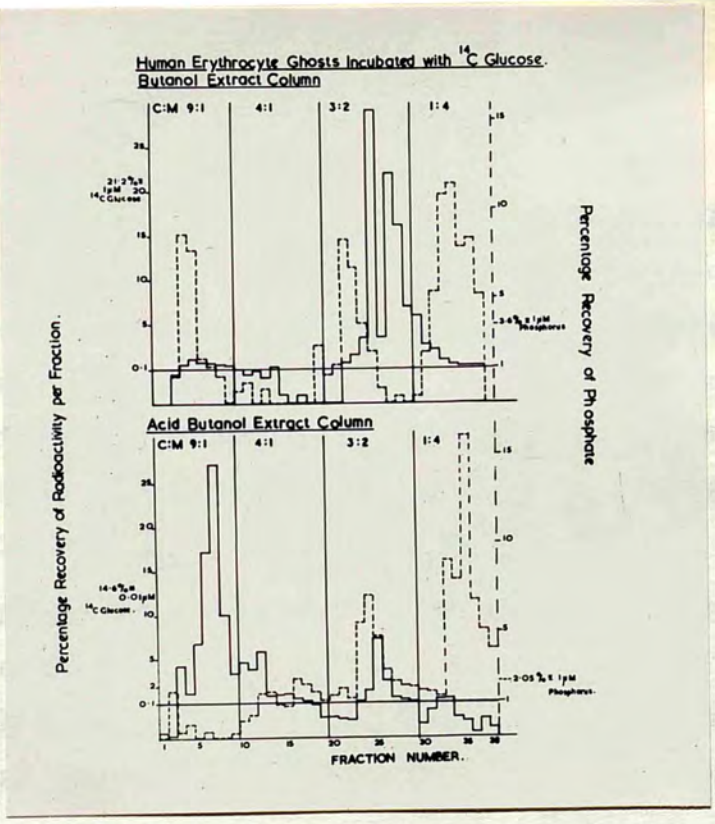
Two portions of red cell ghosts were used in an experiment to compare the results from butanol and acid-butanol extractions with those of chloroform-methanol and acid-chloroform-methanol.

Vol. Ghosts	Activity of Added ^{14}C Glucose	Radioactivity in Butanol	Radioactivity in Acid-Butanol
65.0 ml.	2,632,500 cts/min.	1,764,000 cts/min.	800,932 cts/min.

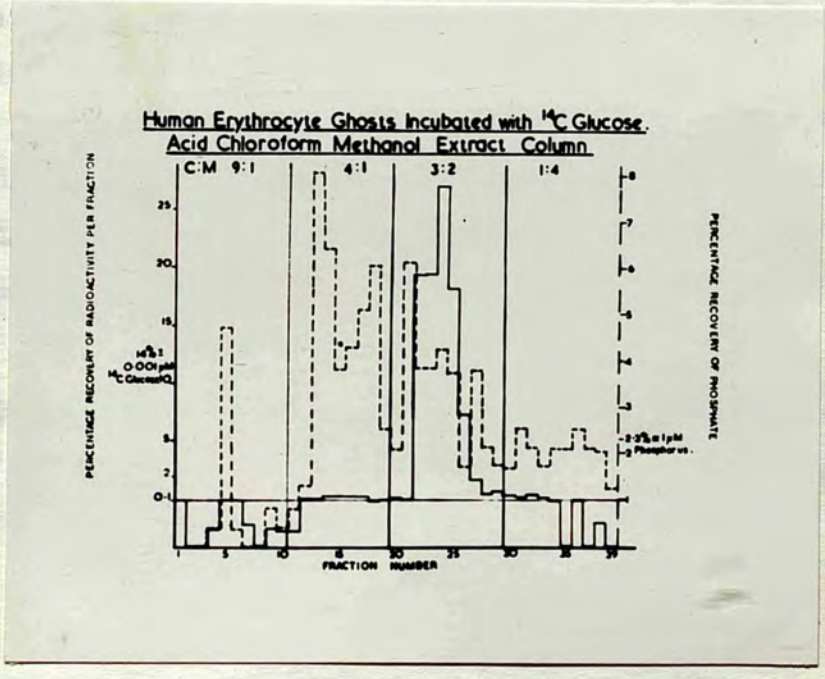
Vol. Ghosts	Activity of Added ^{14}C Glucose	Radioactivity in C - M	Radioactivity in Acid-C-M
65.0 ml.	2,632,500 cts/min.	3,101,000 cts/min.	432,000 cts/min.

The excess recovery of radioactivity in the acid-C-M extract was probably the result of inhomogeneities in the solvent system consisting of an upper phase, a lower phase and an inter-phase. Radioactive counts for each phase were calculated and the total figure is given. The total extract was used for the column chromatography.

Graphs 3 and 4 show the results of column chromatography of the extracts. Graph 3 is similar to the previous Graph 1, and the results are a good correlation. Graph 4 shows the



Graph 3. Silicic acid/Hyflo columns of butanol and acid:butanol extracts. The percentage recovery of radioactivity and the percentage recovery phosphate per fraction are represented by the continuous and broken line graphs respectively.



Graph 4. Silicic acid/Hyflo column of the acid:chloroform: methanol extract. The percentage recovery of radioactivity and the percentage recovery phosphate per fraction are represented by the continuous and broken line graphs respectively.

column chromatograph of the acid-C-M extract and there was only one region of radioactivity corresponding to that of free glucose elution. It was most probable that the formation of a three-phase system in the chloroform-methanol-acid extract resulted in the dissociation of any glucose-lipid complexes. This solvent system was considered unsuitable for further experiments.

Certain fractions of the acid butanol column were selected, some for gas chromatography analysis (Dr. Hitchcock et al, Unilever, Sharnbrook) and others for inositol estimations (Dr. Nixon, St. Mary's Hospital).

Gas Chromatography Analysis

Acid-Butanol column samples:	32 - 33	A
	7	B
	5	C

The samples were evaporated to dryness and refluxed with 1 ml. of 10% methanolic sodium hydroxide for 90 minutes. They were then concentrated, acidified and extracted with ether. The concentrated ether solution was treated with fresh diazomethane for 10 minutes when the volume was reduced to 100 μl. Aliquots of these solutions were analysed by gas liquid chromatography.

'A' contained approximately 400 μg. of fatty acids, while 'B' and 'C' contained about 50 μg. 'A' was not radioactive, while 'B' and 'C' were. The percentages of fatty acids in each are shown in the following table :-

Fatty Acid	A %	B %	C %
18:n			24
18:2	14		
18:1	22	24	
18:0	17	12	13
16:0	33	52	49
14:0	2	8	8
X	7		
Y	5		
Z		4	6

X, Y and Z are unknowns.
18:n is polyhydroxy 18 carbon fatty acid.
18:2 is dihydroxy 18 carbon fatty acid.
18:1 is monohydroxy 18 carbon fatty acid.

'B' and 'C' contained no detectable radioactivity in the fatty acids which showed that the radioactivity of the sample from the column was not the result of metabolic incorporation of C^{14} into the fatty acids of the lipids. Previous thin-layer chromatography of 'B' and 'C' on small thin plates, which were subsequently charred, showed the radioactivity of 'B' and 'C' had a mobility, and therefore polarity, similar to that of phosphatidyl ethanolamine, but they did not exhibit the same colour characteristics on charring.

Inositol Estimations

The following samples were prepared for inositol estimations :-

Acid-Butanol column fraction 11,
Acid-Butanol column fractions 17 - 21.

The pooled fractions were vaporated to dryness, then refluxed with 6 N hydrochloric acid for 18-24 hours. The resulting acidity was neutralised by the repeated process of water dilution and evaporation until the pH of the

samples was of the order 5 - 7. The microbiological assay was carried out by Dr. D.A. Nixon using Kloekera brevis. Results:-

Fraction 11 contained approximately 1.06 μg. inositol.

Fractions 17-21 contained approximately 5.0 μg. inositol.

Thus, Fraction 11 had approximate concentrations of 2 nM glucose, 6.0 nM inositol and less than 0.5 μM phosphorus. Fractions 17-21 contained negligible glucose, 30 nM inositol and 2.5 μM phosphorus. The order of the phosphate-inositol ratio in fractions 17-21 did not indicate the presence of phosphoinositides in these fractions.

Acid Butanol as an Extracting Solvent

The results showed that acid-butanol extracted a large proportion of radioactivity which gave three regions of radioactivity on silicic acid column chromatography. The region eluted in solvent C-M 4:1 (v/v) was separated into two peaks of radioactivity on subsequent thin-layer chromatography. The second of these, of Rf value 0.1 - 0.2, was not attributable to free glucose; however, it was found that the high concentration of this material exceeded the calculated requirements for saturation of the glucose carrier. An experiment was performed in which glucose and acid-butanol alone were subjected to the same chemical treatment as the acid-butanol extract of ghosts incubated with ^{14}C glucose. Thin-layer chromatography and subsequent radioactive scanning of the acid-butanol + ^{14}C glucose material showed that there were two regions of radioactivity similar to those previously obtained with the ghost extract. The second peak in the artifact material was relatively smaller than that of the ghost acid extract and was thought to be the result of the chemical reaction of glucose with higher alcohols under acidic conditions to give compounds such as semi-acetals, $R\text{-}CH_2\text{-}CH(OH)OC_nH_{2n+1}$. The reaction is very slow and it was unlikely that all the material of the second radioactive peak in the acid ghost extract was the semi-acetal compound.

But separation of the semi-acetal compound from other material of the same R.F. value was not achieved and the use of acidified extraction solvents was terminated.

Isopropanol-Chloroform Extracts

When red cell stroma were extracted with an isopropanol-chloroform 11:7 v/v solvent (Rose & Ocklander, 1965) the lipid yield was high and there was no contamination of the extract with haem pigment. Similar results were recorded when an extraction solvent of isopropanol-ethyl acetate 11:7 v/v was used. An experiment to compare the radioactive sugar distribution in the subsequent silicic acid chromatography solumns of these extracts was performed.

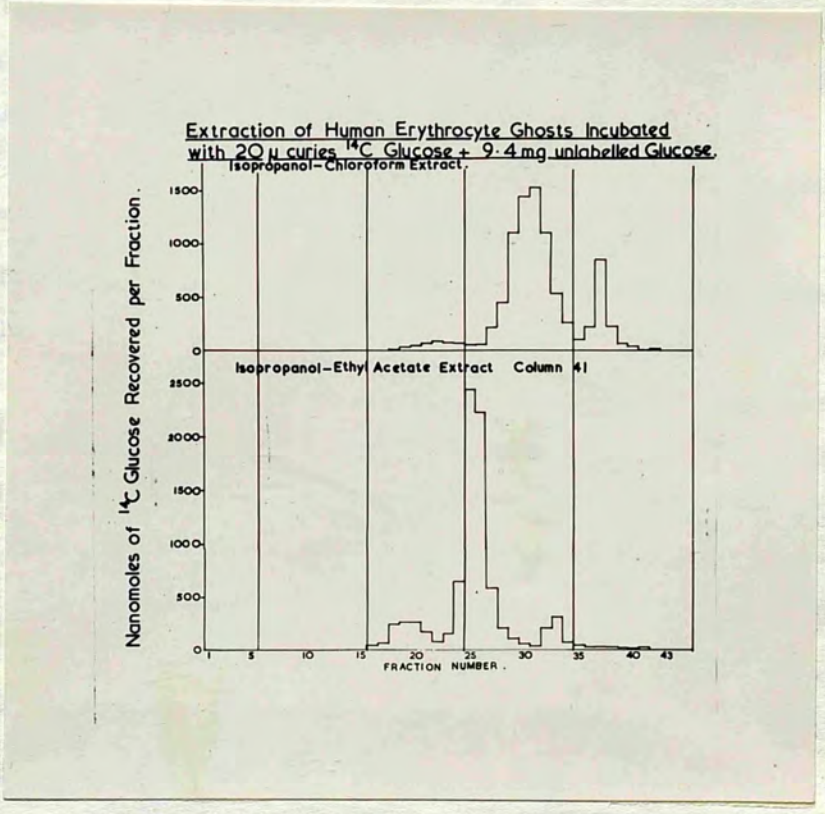
Vol. Ghosts	Total Activity ^{14}C	Recovery ^{14}C in isop/$CHCl_3$
80.0 ml.	2,625,000 cts/min.	1,504,000 cts/min.

Vol. Ghosts	Total Activity ^{14}C	Recovery ^{14}C in isop/Eth.Ac.
80.0 ml.	2,625,000 cts/min.	1,563,000 cts/min.

The weight of ^{14}C glucose in the stock solution was very small, 0.03 mgm, so that only small quantities of radioactive lipid complex material could be expected. To increase the latter, ^{12}C glucose was added to the ghost incubation media to give a final concentration of 9.48 mgm of sugar. Both extracts were prepared, after solvent evaporation, for chromatography on silicic acid-Hyflo columns. The distribution of radioactive sugar in the eluent fractions is represented on Graph 5. Both columns showed three main regions of radioactivity.

Isopropanol-Chloroform Column :-

Fractions	19-23	28-33	36-38
eluent C-M	4:1	3:2	1:4



Graph 5. Silicic acid/Hyflo columns of isopropanol: chloroform, 11:7 and isopropanol : ethyl acetate, 11:7 extracts. The recovery of radioactive glucose per fraction is represented.

Isopropanol-Ethyl Acetate Column :-

Fractions	17-21	23-28	33-35
eluent C-M	4:1	3:2	1:4

The radioactivity in the 3:2 eluent was where free glucose is eluted. Material from all three regions of each column was chromatographed on silica gel G plates in chloroform-methanol-water 80:30:3. Fractions 20-21, 31 and 35 were selected from the isopropanol-chloroform column, and Fig. 8 shows the chromatographed plate developed in a saturated iodine tank. All three samples were heterogenous, 20-21 and 31 streaking from the origin to Rf 0.6. Sample 35 contained less material than the other two samples, with most material at or near the origin. The lipid standards also appeared to be heterogenous. The radioactive scans of all three samples showed the presence of free glucose, but 20-21 and 35 appeared to have two maxima in the peak region. Sample 31 was only free glucose. C_1 and C_2 were solvent controls where only redistilled chloroform was applied to the plate.

Fig.9 shows the same plates developed with ninhydrin. Samples 20-21 and 35 gave the characteristic purple colour reaction, while in sample 31 there was no detectable colour. Sample 20-21 streaked with colour from the origin to Rf value 0.5, while in sample 35 the colour was at or near the origin. The phosphatidyl ethanolamine standard also streaked from the origin to Rf 0.4. The impurity of commercially-available standards limited the analysis of both the iodine and ninhydrin developed plates.

Fractions 18, 25 and 33 from the isopropanol-ethyl acetate column were chromatographed in the chloroform-methanol-water system, 80:30:3, on silica gel G thin-layer plates.

Fig.10 shows the iodine development of the chromatographed plate. All fractions were heterogenous, with less material in 25 than in 18 and 33. Sample 18 had a similar development to that of the standard phosphatidyl ethanolamine sample. Fraction 25 was similar to the sphingomyelin standard, while Fraction 33 developed strongly in a region of Rf. value 0.2 - 0.3 which corresponded to several regions in the standard lipids. The radioactive scan of all three fractions showed the presence of free glucose, but in sample 18 the radioactivity peak began nearer the origin than free glucose while in Fraction 35 it extended over the region of the free glucose sample.

Fig.11 shows the ninhydrin reaction of the same plate. The large spot of Rf. value 0.45 in sample 18 corresponded with the same spot in the phosphatidyl ethanolamine sample. Fraction 25 had no detectable reaction, while Fraction 33 gave a strong colour reaction at Rf. 0.15 which corresponded to a second region in the phosphatidyl ethanolamine standard, and the same region was also detectable in the phosphatidyl inositol standard.

It appeared that the radioactivity in the regions on either side of the free glucose peak of silicic acid column chromatography may not have been free glucose alone but that the solvent system C-M-H_2O, 80:30:3, used for thin-layer plate chromatography encouraged the dissociation of any complex material. The material other than free glucose could have formed outside the red cells during the process of evaporation of the lipid extract. Silicic acid/Hyflo column chromatography was performed on two extracts (a) where the ghosts + ^{14}C glucose were incubated before lipid extraction, (b) the ghosts were extracted with lipid solvent and the ^{14}C glucose was added to the extract. Graph 6 shows the distribution of sugar radioactivity in the column chromatographs of the two extracts. The control experiment

Fig. 8. Thin layer chromatogram of Fractions 20 and 21, Fraction 31 and Fraction 35 of silicic acid column of the isopropanol:chloroform extract. The plate was chromatographed in C:M:H_2O, 80:30:3 and developed in a saturated iodine tank.

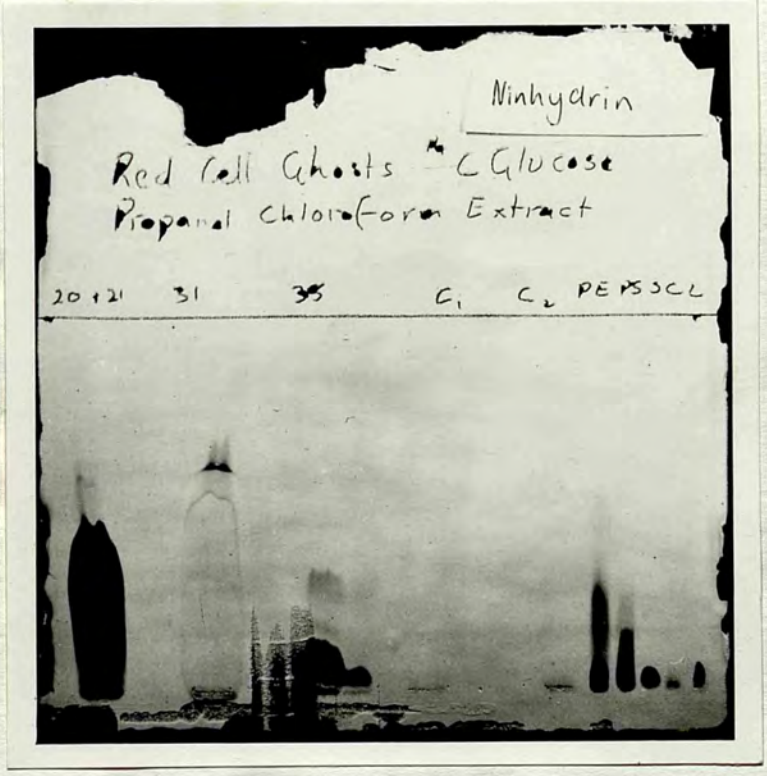


Fig. 9. As Fig. 8 but the plate was developed with ninhydrin.

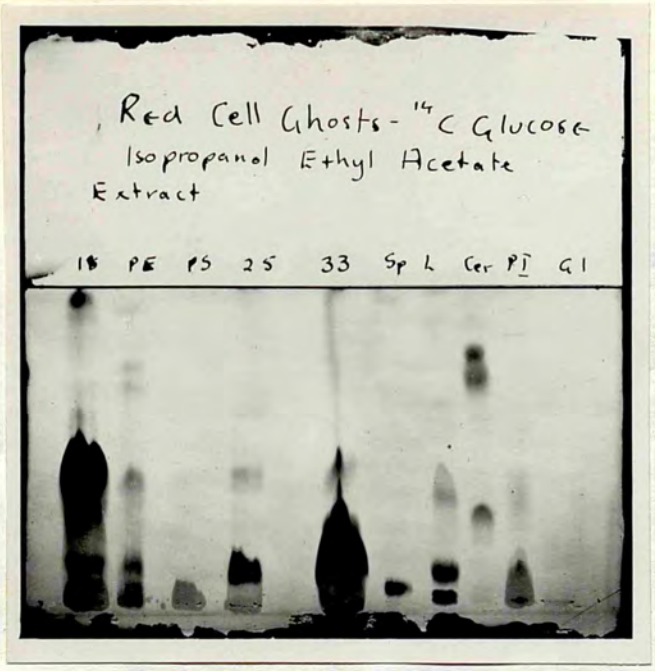


Fig.10. Thin layer chromatogram of Fraction 18, Fraction 25 and Fraction 33 of the silicic acid column of the isopropanol:ethyl acetate extract. The plate was chromatographed in C:M:H_2O,80:30:3 (by vol) and developed in a saturated iodine tank.

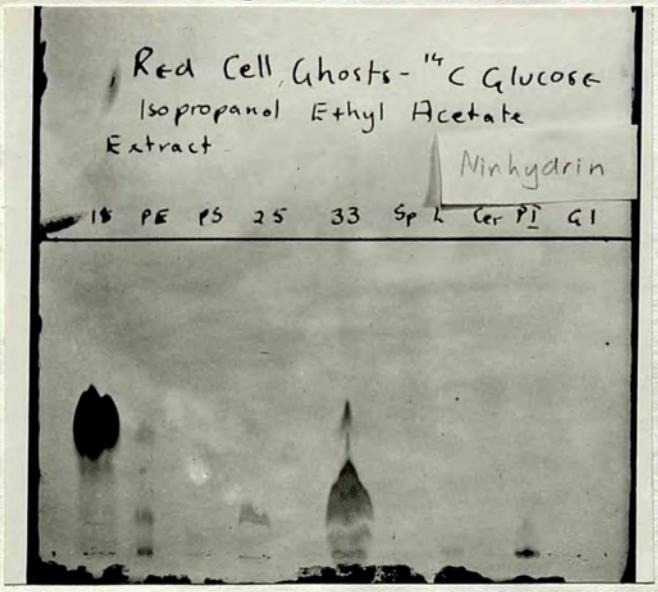
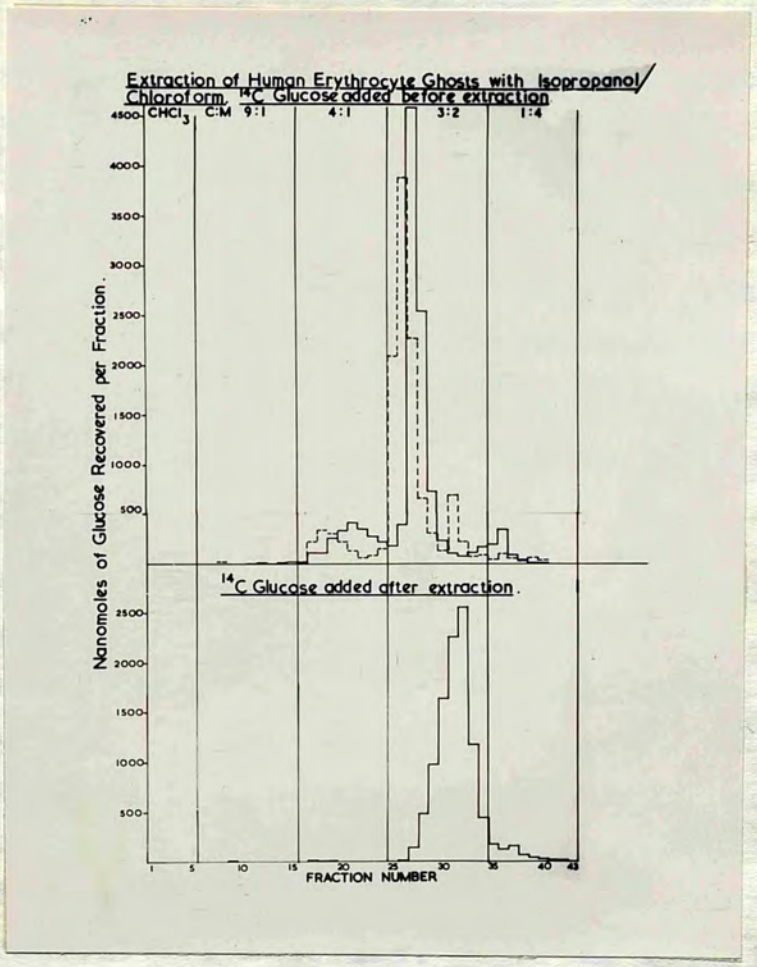


Fig.11. As Fig.10 but after development with ninhydrin.



Graph 6. Silicic acid/Hyflo columns of isopropanol: chloroform 11:7 extracts. The ^{14}C glucose was added before and after lipid extraction. The radioactive sugar per fraction is represented.

gave only one region of radioactivity in the free glucose eluent. While two identical experiments of the ghosts incubated with ^{14}C glucose before lipid extraction chromatographed to give the three regions of radioactivity.

The material in Fractions 17-20 of the isopropanol-chloroform silicic acid column dissolved in carbon tetrachloride and the infra red spectra was similar to that of a phosphoinositide lipid prepared from rat brain (Fig.12), but the concentration of the material was very low and the phosphoinositide sample was impure, being contaminated with other [illegible]lipids, in particular with cerebroside and sphingomyelin.

Chloroform Insoluble Radioactivity

It was observed that when the isopropanol-chloroform extract of the ghosts + ^{14}C glucose incubation was evaporated to dryness and redissolved in chloroform for column chromatography, there was often a residue of the dried material which was insoluble in the chloroform. A second extraction was made of this residue material with dimethyl formamide and column chromatography was performed on the soluble radioactive material. In one experiment the following data was recorded :-

Vol. of Ghosts	Activity of ^{14}C Added	Recovery of cts. in Isop.-Chloro
50.0 ml.	1,444,625 cts/min.	1,386,560 cts/min.

After evaporation of the isopropanol-chloroform under reduced pressure and in a nitrogen atmosphere, the residue material was dissolved in 2.0 ml. of redistilled chloroform. The solution was centrifuged for 5 minutes at 3,000 r.p.m. and the supernatant removed. When the remaining residue was completely dry, 2.0 ml. of redistilled dimethyl formamide were added to give a second radioactive extract. Radioactive counts were calculated for the two extracts.

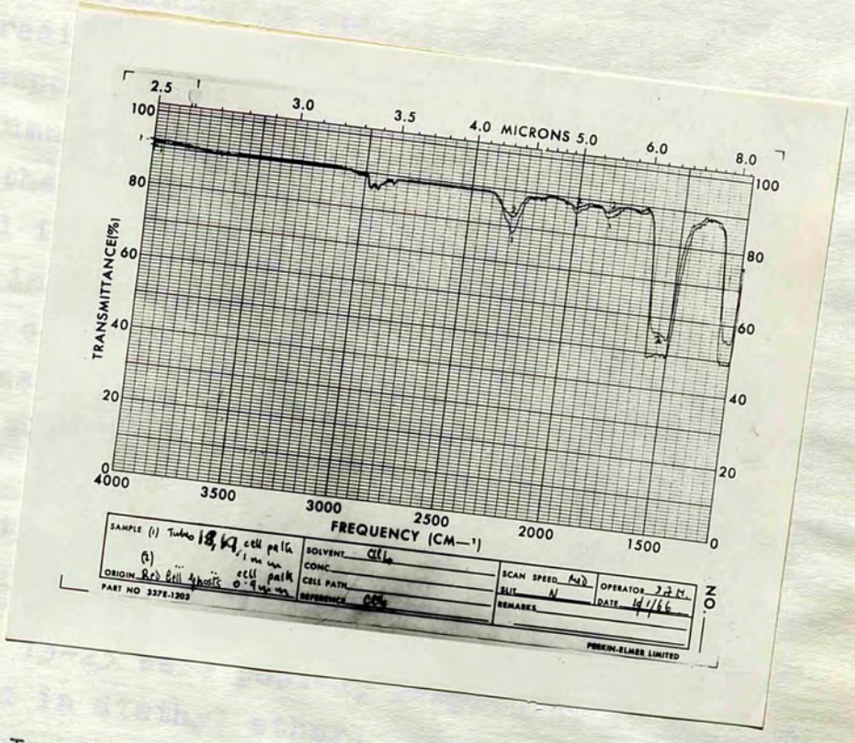
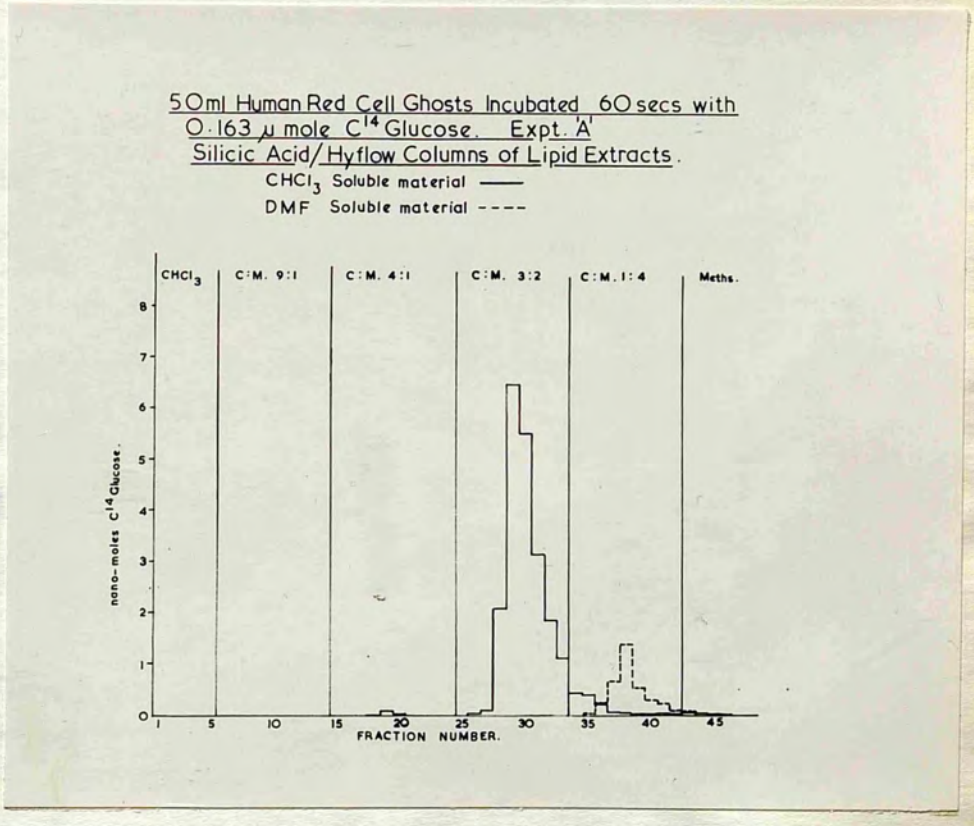


Fig.12. Infra red spectra of the material from Fractions 18 and 19 of the silicic acid column of the isopropanol:chloroform extract.

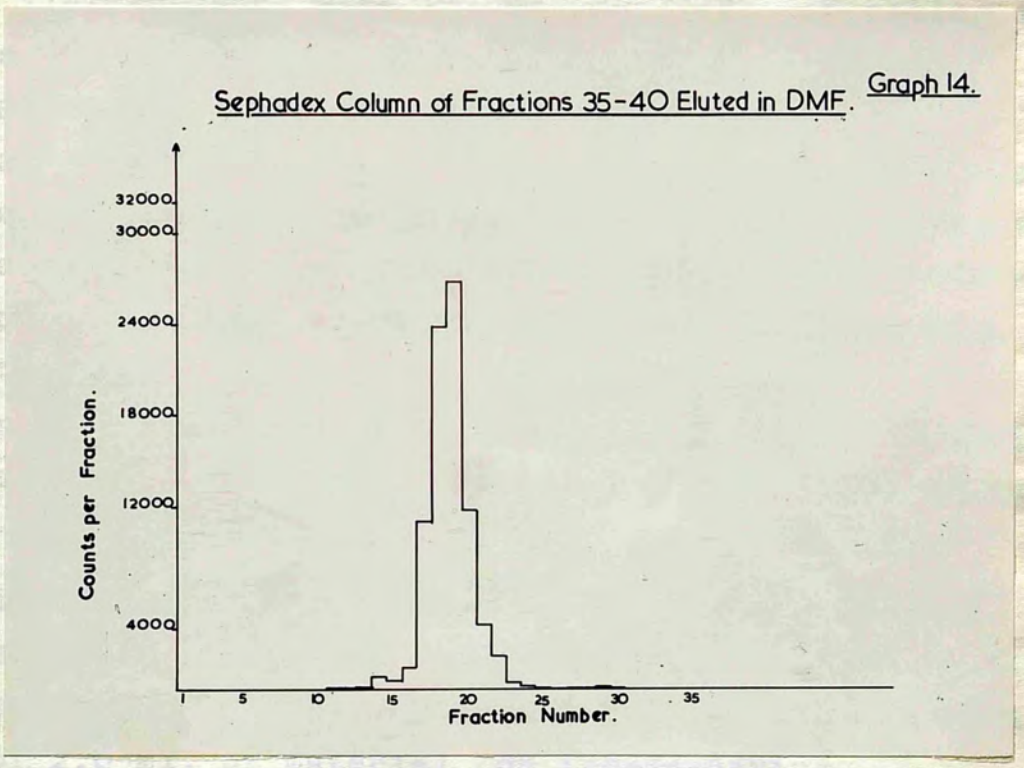
Chloroform 536,720 cts/min.
Dimethyl Formamide306,120 cts/min.

Both extracts were chromatographed on silicic acid/hyflo columns and the radioactivity distribution in the eluents recorded. (Graph 13). The chloroform extract gave the same radioactive distribution previously recorded although the radioactivity region in the 4:1 C-M solvent was reduced while that in 1:4 was increased, and there was a large quantity of free glucose in the 3:2 solvent. The dimethyl formamide-extracted material gave only one region of radioactivity in the 1:4 C-M solvent, Fractions 35-40 corresponding to the latest peak in the chloroform extract column chromatograph. The Fractions 35-40 were pooled and the chloroform-methanol evaporated under reduced pressure and in a nitrogen atmosphere. The residue was redissolved in dimethyl formamide and applied to a Sephadex G10 column swollen in dimethyl formamide. The bed volume was 100 x 0.5 cm. and 2.5 ml. fractions were collected. The radioactive distribution is represented in Graph 14. There was only one region of radioactivity in Fractions 13-23. Free glucose applied to the same column was eluted in Fractions 30-37.

Fractions 13-23 were pooled, evaporated to dryness and redissolved in diethyl ether. Radioactive counting of the solution showed most of the counts were dissolved in the ether. The ethereal solution was then shaken up with distilled water and samples of the resulting water and ether fractions taken for count estimations. The results showed that all the radioactivity appeared in the water fraction. Thin-layer chromatography of both fractions on silica gel G plates run in C-M-H_2O, 80:30:3 (by vol) confirmed this. All the radioactivity of the water fraction chromatographed to give a radioactive peak only at the free glucose R_f position. Unfortunately, there was very little material at this stage



Graph 13. Silicic acid/Hyflo columns of the chloroform and dimethyl formamide soluble radioactivity of the isopropanol:chloroform 11:7 extract.



Graph 14. Sephadex G10 column of Fractions 35-40 of the silicic acid/Hyflo column of the dimethyl formamide soluble material. The radioactive counts per fraction are represented.

and the ether fraction did not develop with either iodine or ninhydrin detection. Since it appeared that later eluted radioactivity material was only soluble in more polar solvents it was decided to see if it was possible to purify the lipid-glucose material by partially separating it from excess ^{14}C glucose and other lipid material with a series of extracting solvents before any column chromatography was performed. The solvent series used was the same as that of Handa et al. in their isolation of human erythrocyte glycolipids. (Fig.13). Ghosts were freeze-dried before the initial extraction of lipid material with methanol-ether 1:1. In a series of experiments, comparisons were made of results from this method with those of previous ones. A group of experiments was set up as shown in Fig.14.

The quantities of ghosts used in experiments B and C were limited by the method of freeze-drying. The freeze-dry apparatus consisted of twelve phials, each of which carried a maximum of 2.0 ml. of material for freeze-drying. Six phials were used for each of B and C experiments, each containing 1.8 ml. of ghosts and 0.2 ml. of stock ^{14}C glucose solution. The concentration of sugar in the stock solution was increased by the addition of 9.0 mgm. of ^{12}C glucose to give a final concentration of 9.078 mgm. of glucose in 5.0 ml. In experiment A, 2.0 ml. of the stock solution were incubated with the ghosts, and in experiments B and C 1.2 ml. to give final concentrations in each experiment of :-

A - 20 μmoles of glucose
B - 12 μmoles of glucose
C - 12 μmoles of glucose

In experiments B and C the ghosts and glucose were incubated with continual mixing for one minute in the phials before positioning in the freeze-dry apparatus. When

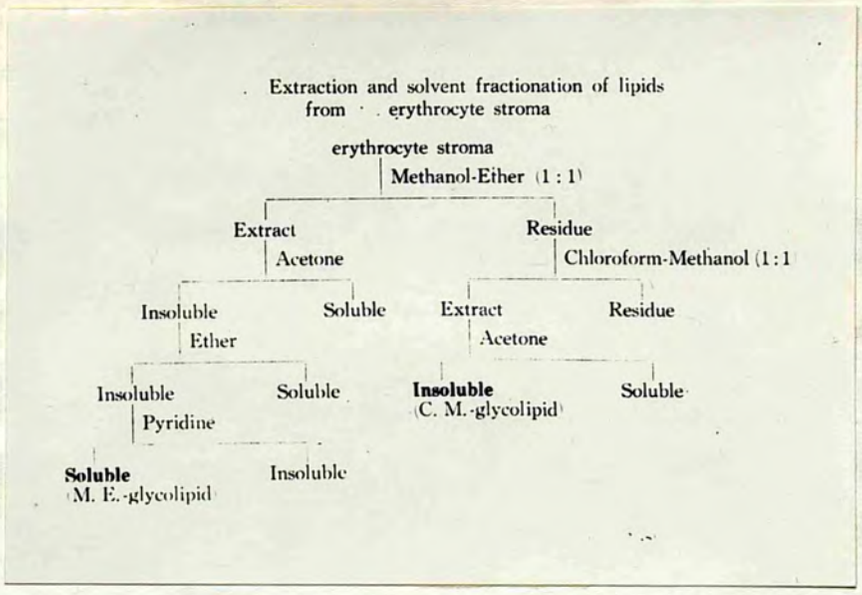


Fig.13. The extraction procedures for the isolation of methanol:ether glycolipids as described by Handa et al (1963).

Expt.	ml. of Ghosts.	C^{14} Glucose	C^{12} Glucose.	Method of extraction.
A	30 ml.	0·0292 mgm.	3·6 mgm.	Isopropanol/$CHCl_3$ after incubation with C^{14} Glucose for 1 min.
B	10·8 ml.	0·0175 mgm.	2·016mgm.	Freeze dried after incubation for 1 min. with C^{14} Glucose then extracted with Isopropanol/$CHCl_3$.
C	10·8 ml.	0·0175 mgm.	2·016mgm.	Freeze dried after incubation for 1 min. with C^{14} Glucose then extracted with Methanol/Ether 1:1.

Fig.14. A group of experiments to compare results from :-

A. Isopropanol:chloroform extraction of aqueous preparations.

B. Isopropanol:chloroform extractions of freeze-dried preparations.

C. Methanol:ether extraction of freeze-dried preparations.

completely free of water, the six phials of experiment B were thoroughly washed out with isopropanol-chloroform 11:7 v/v and those of experiment C with methanol-ether 1:1 v/v. Experiment A was similar to previous experiments where the isopropanol-chloroform 11:7 v/v was the extraction solvent on the wet ghosts. The recovery of ^{14}C counts in all three experiments was :-

Experiment	Activity of ^{14}C added	Recovery of ^{14}C in Iso/$CHGL_3$
A	1,435,000	1,256,360
B	861,000	492,000

Experiment	Activity of ^{14}C added	Recovery of ^{14}C in M-E
C	861,000	995,350

The isopropanol-chloroform extracts of A and B were evaporated to dryness under reduced pressure in a nitrogen atmosphere. The residue was redissolved in redistilled chloroform for application to silicic acid/hyflo columns. There was no precipitate in the chloroform extract of A and B on this occasion.

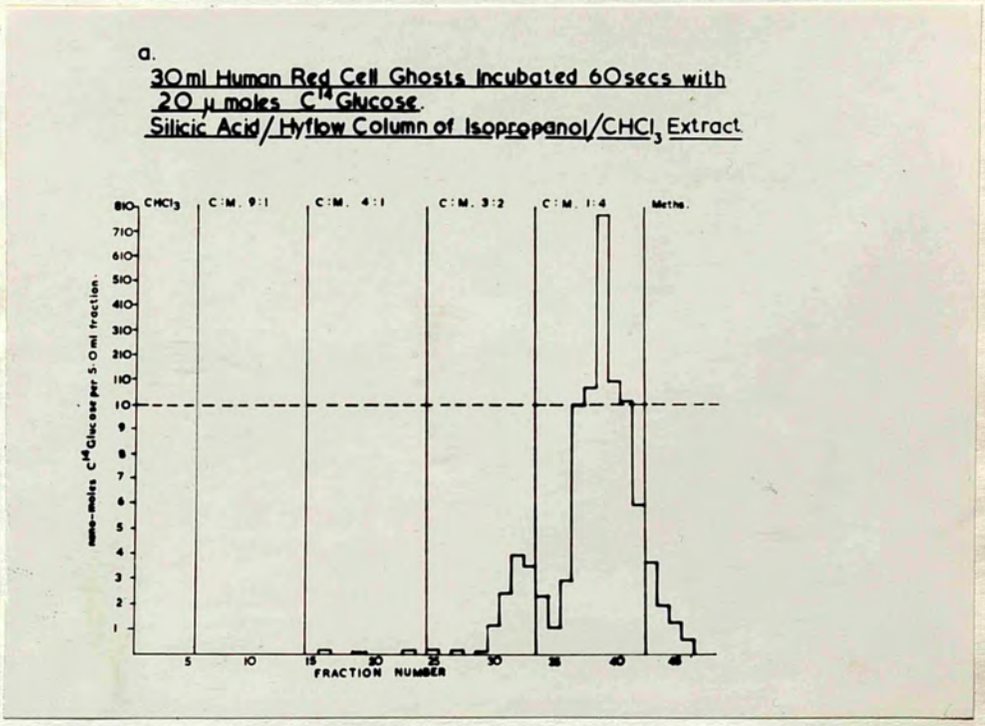
The methanol-ether of experiment C was evaporated under reduced pressure in an atmosphere of nitrogen and the residue shaken up with acetone. The majority of the residue was insoluble in the acetone, which was subsequently decanted and counted for radioactivity. The insoluble residue was extracted with diethyl ether when again only a proportion of the material dissolved. The ethereal extract was centrifuged for five minutes at 3,000 r.p.m. when the supernatant was removed and the radioactivity estimated. The residue was redissolved in pyridine, no insoluble material remained. The recovery of radioactivity in each of the solvents was :-

Acetone	45,750 cts/min.
Diethyl Ether	48,500 cts/min.
Pyridine	442,000 cts/min.

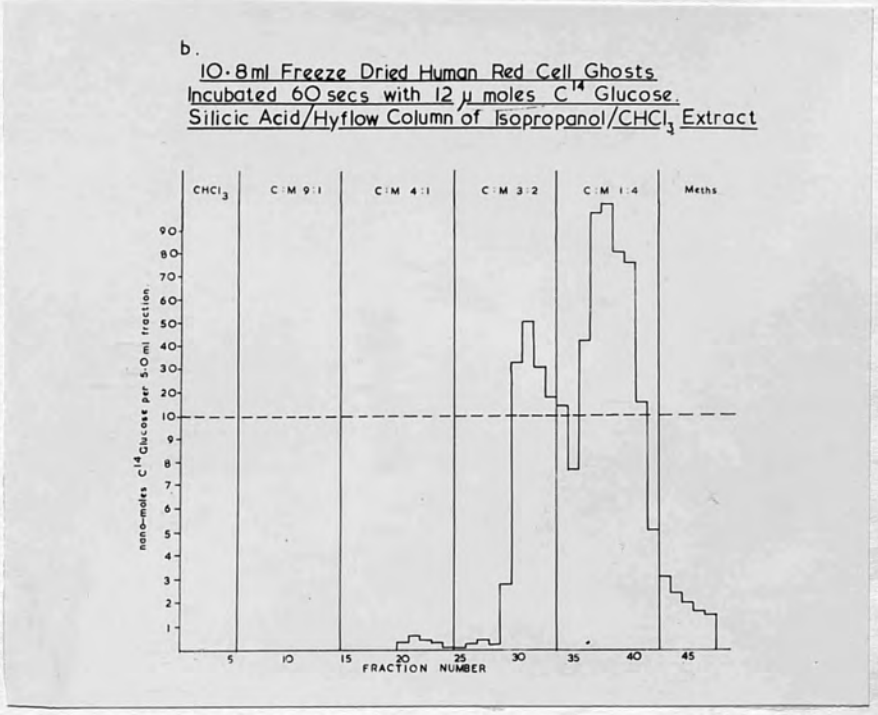
The pyridine was evaporated under reduced pressure in a nitrogen atmosphere and the residue redissolved in redistilled chloroform for application to a silicic acid/hyflo column. All the residue was soluble in the chloroform. Graphs 15, 16 and 17 show the elution of radioactivity in the isopropanol-chloroform extracts of experiments A and B, and the pyridine extract of experiment C, respectively.

In A and B there were the three usual peaks of radioactivity although that in C-M 4:1 was very small. In column C there was only one region of radioactivity, viz. in the 1:4 eluent fractions 35-40. Since this result was similar to that previously obtained for the dimethyl formamide-soluble material of an isopropanol-chloroform residue, the remainder of some dimethyl formamide (DMF) material, which had been stored in the refrigerator was dried and treated with the series of solvents. All the counts appeared in the pyridine fraction, with negligible quantities in the acetone and diethyl ether fractions. The pyridine fraction was chromatographed on silicic acid column and again the radioactivity appeared only in Fractions 35-40 of the C-M 1:4 eluent. Thus the DMF. and pyridine materials were the same.

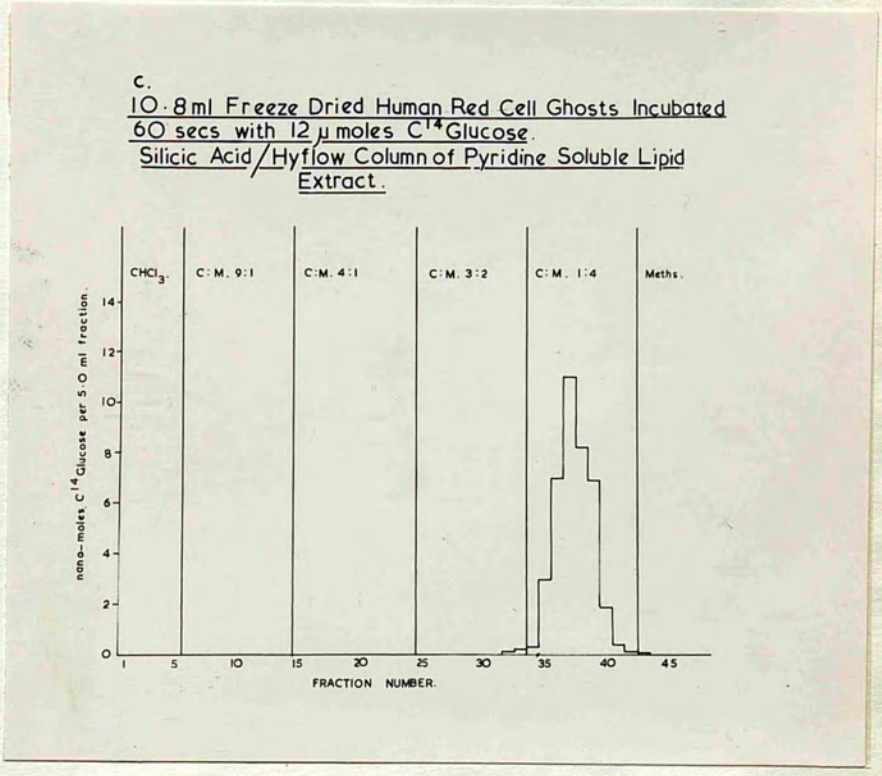
A sample of stock ^{14}C glucose was also subjected to the glycolipid solvent extraction process and the pyridine fraction prepared for silicic acid column chromatography. There were few counts in the pyridine solvent, most of them had dissolved in the acetone and diethyl ether solvents, but there was sufficient radioactivity to plot the fractional distribution of radioactivity in the chromatographic column, as shown in Graph 18. All the radioactivity present appeared



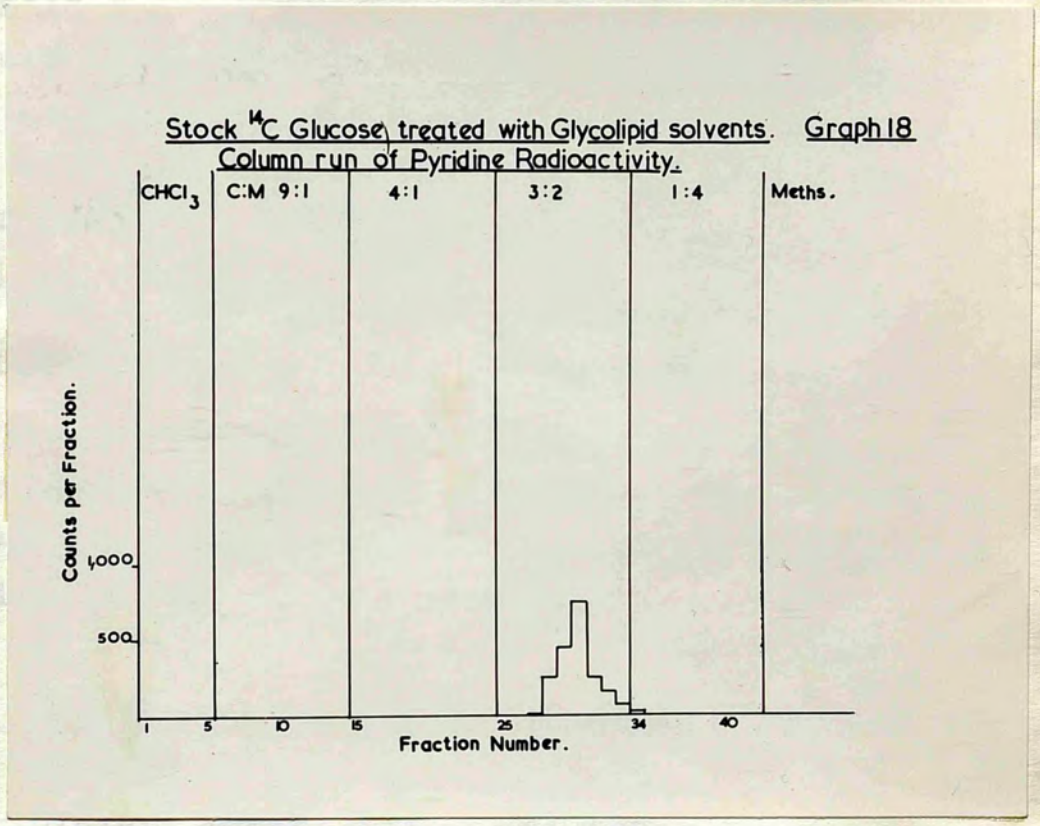
Graph 15. Silicic acid/Hyflo column of the isopropanol: chloroform 11:7 extract of an aqueous preparation of ^{14}C glucose and ghosts. The radioactive sugar per fraction is represented.



Graph 16. Silicic acid/Hyflo column of the isopropanol: chloroform 11:7 extract of a freeze-dried preparation of ^{14}C glucose and ghosts. The radioactive sugar per fraction is represented.



Graph 17. Silicic acid/Hyflo column of the pyridine soluble material of a methanol:ether 1:1 extract of a freeze-dried preparation of ^{14}C glucose and ghosts. The radioactive sugar per fraction is represented.



Graph 18. Silicic acid/Hyflo column of the pyridine soluble radioactivity of a methanol:ether extract of stock ^{14}C glucose. The radioactive counts per fraction are represented.

in the 3:2 eluent in Fractions 28-34.

An experiment similar to that of C of the previous group was performed. The stock glucose solution added to the ghosts gave a final concentration of 1.2 μ moles. of glucose. Each phial was cooled in ice before the introduction of cold ghosts and radioactive glucose. The ghosts and sugar were incubated for one minute, with thorough mixing before the phial was positioned on the freeze-dry apparatus. When dry, the powdered ghosts were carefully extracted from the phials with methanol-diethyl ether 1:1 v/v. The recovery of counts in the M-E fraction was :-

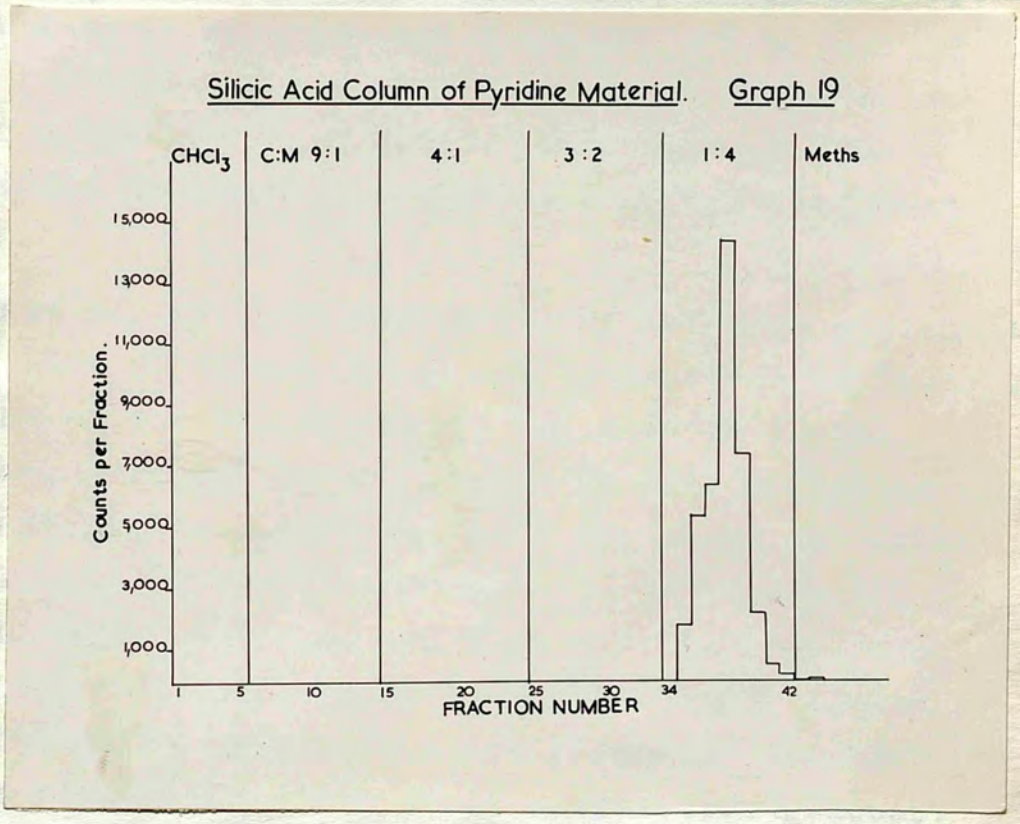
Activity ^{14}C Added	Activity ^{14}C Removed M-E
896,250 cts/min.	411,072 cts/min.

After evaporation of the methanol-ether under reduced pressure in a nitrogen atmosphere, the residue was extracted with the series of solvents and the quantity of radioactivity in each solvent calculated.

Solvent	Total Cts/Min.
Acetone	45,120
Diethyl Ether	22,995
Pyridine	347,000

The pyridine material was prepared for silicic acid hyflo column chromatography and Graph 19 represents the subsequent distribution of radioactivity in the column eluents. All the radioactivity was eluted in fractions 35-42 with the C-M 1:4 solvent.

The Fractions 35-42 were pooled and evaporated to dryness under reduced pressure and an atmosphere of nitrogen, in readiness for application to silica gel G thin-layer plates. Two solvent systems were used for separation (a) C-M-H_2O 80:30:3, (b) dimethyl formamide. A sample of stock ^{14}C glucose was also applied to the two



Graph 19. Silicic acid/Hyflo column of the pyridine soluble material of a methanol:ether 1:1 extract of a freeze-dried ^{14}C glucose and ghosts preparation. The radioactive counts per fraction are represented.

plates. When the solvents had dried from the plates each was scanned for radioactivity. Fig.15 shows the result. In the C-M-H_2O solvent radioactivity of both samples remained at the origin, but in the dimethyl formamide solvent the pyridine-soluble material gave two regions of radioactivity - a small one at the origin, which corresponded to the scan of free glucose, and a larger second peak at Rf 0.85. Although the plate failed to develop in iodine or with ninhydrin, a spot of Rf 0.85 in the pyridine-soluble material was visible under ultra-violet light.

Since not all the pyridine material had been applied to the plates, some of it was dried and redissolved in diethyl ether. Most of the radioactivity was soluble in the diethyl ether, but when it was shaken up with distilled water and radioactive counts taken of the ether and water fractions all the radioactivity appeared in the water. The fractions were separated and the ether material was dried, some was re-chromatographed on thin layer plates and some redissolved in pyridine and incubated for one hour at room temperature with stock radioactive glucose. The resulting solution was dried, redissolved in chloroform and applied to a thin-layer plate which was chromatographed in a dimethyl formamide solvent system.

The radioactive scan results are summarised in Fig.16. The absence of radioactivity in the ether fraction, after shaking up with distilled water, was verified by the radioactivescan of the fraction. The water fraction contained only free glucose at the origin. The material resulting from the incubation of the ether material with radioactive sugar in a pyridine medium chromatographed to give a large peak of free glucose at the origin, but there was also a smaller second peak of Rf value 0.85. Again the material fluoresced under ultraviolet light, as also did a spot of the same Rf value in the non-radioactive

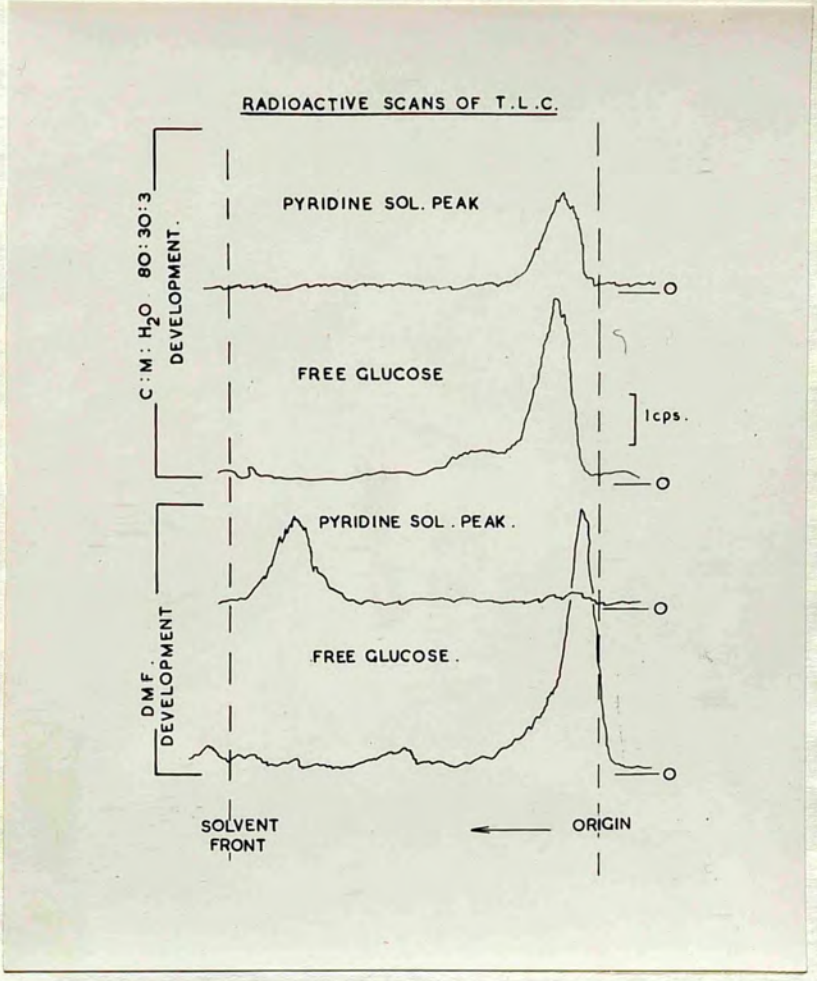


Fig.15. Radioactive scans of Fractions 35-42 of the silicic acid column of the pyridine extract. The plates were chromatographed in dimethyl formamide and C:M:H_2O 80:30:3 solvent systems.

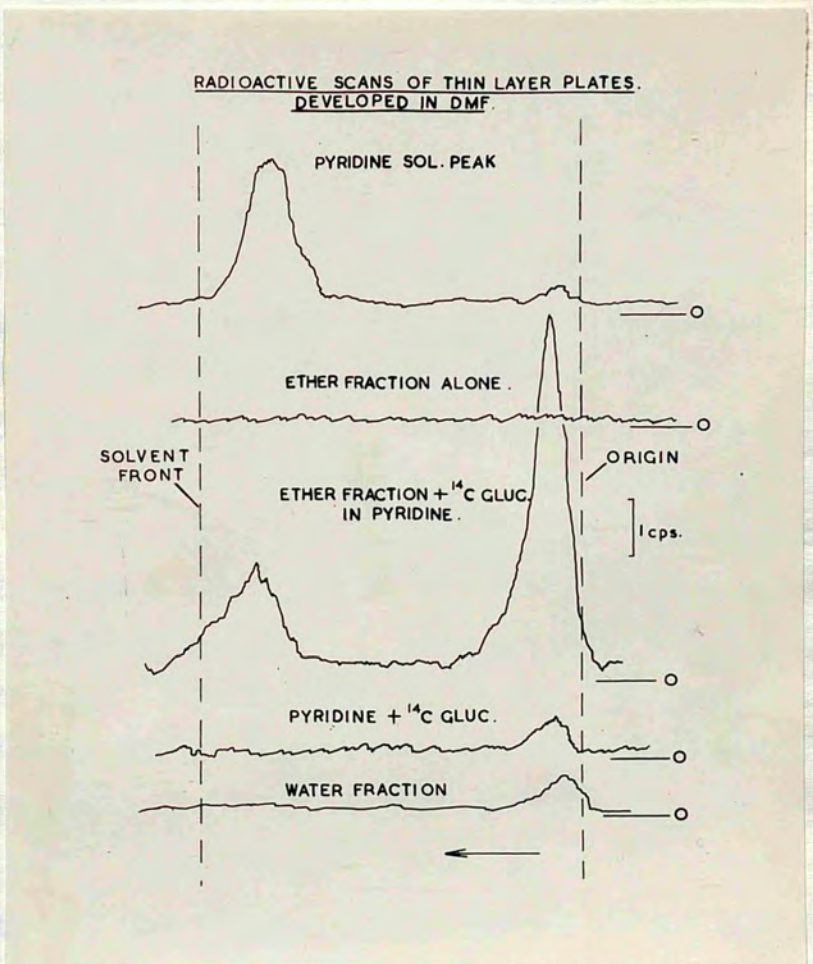


Fig.16. Radioactive scans of Fractions 35-42 of the silicic acid column of the pyridine extract before and after shaking the material up with ether:water and rechromatographing the ether and water phases. The plate was chromatographed in dimethyl formamide.

ether fraction.

Thus the distilled water had split off the glucose from its component of Rf value 0.85, but the complex had reformed on incubation of the same component with fresh radioactive glucose in pyridine. A scan of stock radioactive glucose alone, incubated in pyridine for one hour at room temperature, showed the resulting material gave only one radioactive peak at the origin.

Development of the thin-layer plate in dimethyl formamide suppressed any detection of lipid components with iodine or ninhydrin. The water and ether fractions of the pyridine material were also chromatographed in a C-MH_2O 80:30:3 solvent system. The plate was developed in a saturated iodine tank. (Fig.17.) The ether fraction stained at the origin and at a spot of Rf value 0.3. This spot corresponded to similar spots in phosphoinositol and phosphatidyl ethanolamine. Most of the standard lipids were heterogeneous. The ninhydrin stain was negative. The water fraction, which was shown to contain only free glucose, stained only faintly at the origin in the iodine tank.

The ability of the sugar to recombine with the lipid component in the pyridine medium indicated the possibility of the complex material not actually forming in the red cell membrane. The complex material might have formed in the pyridine solvent as a result of the separate solubilisation of the sugar and lipid components in this solvent. Fig.18 represents the two possibilities.

A control was set up using six phials of the freeze-drying apparatus for each of two experiments :-

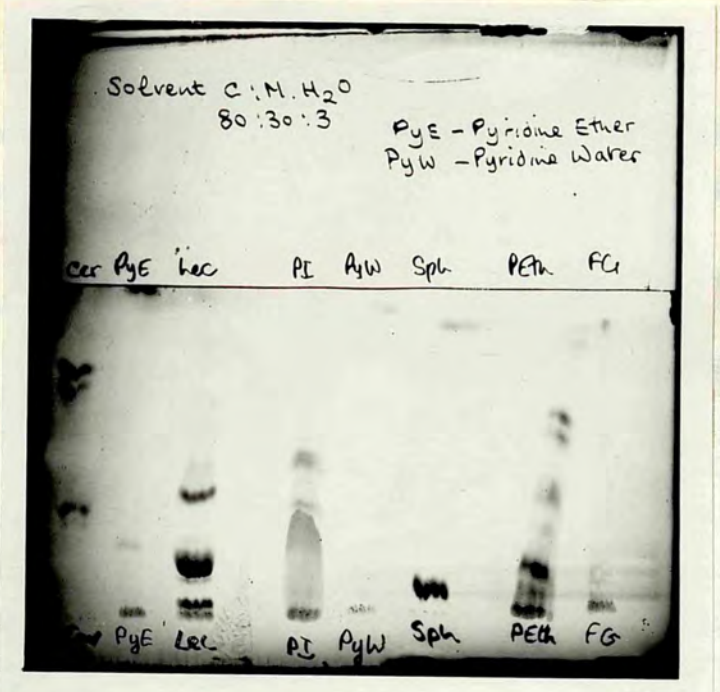


Fig.17. Thin layer chromatograms of Fractions 35-42 of the silicic acid column of the pyridine extract after shaking the material up with ether:water and rechromatographing the ether and water phases. The plate was chromatographed in C:M:H_2O 80:30:3.

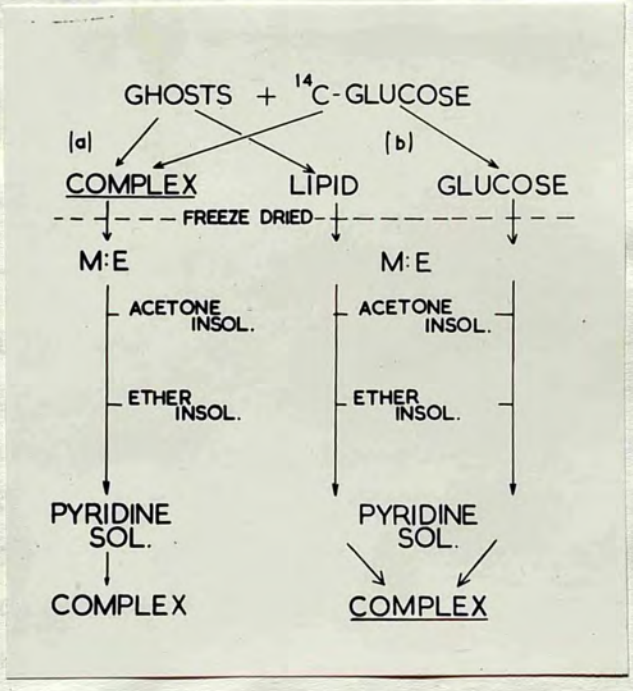


Fig.18. Representation of the possibilities of complex formation (a) within the red cell ghost membrane (b) in the pyridine solvent.

Experiment A

4 phials contained 2.0 ml. of ghosts each.
2 phials contained 0.6 ml. ^{14}C stock glucose each.

Experiment B

All 6 phials contained 1.4 ml. ghosts each, and
0.2 ml. ^{14}C stock glucose each.

The six phials of each experiment were thoroughly extracted with methanol-diethyl ether 1:1 v/v so that in Experiment A the sugar was only introduced to the red cell lipids in the methanol-ether extract.

Both methanol-ether extracts were divided into two equal portions to give four quantities of M-E extract, A_1, A_2, B_1 and B_2. The radioactivity recovery in each was calculated.

Experiment A

Activity ^{14}C Added	Activity ^{14}C A_1	Activity ^{14}C A_2
3,695,250	794,700	777,600

Experiment B

Activity ^{14}C Added	Activity ^{14}C B_1	Activity ^{14}C B_2
3,695,250	911,360	911,000

All four methanol-ether residues were treated with acetone and diethyl-ether, but A_1 and B_1 were finally dissolved in pyridine while A_2 and B_2 were dissolved in dimethyl formamide. The distribution of counts in each solvent was calculated.

Solvent	Counts in A_1	Counts in A_2	Counts in B_1	Counts in B_2
Acetone	146,040	111,230	140,040	149,760
Diethyl Ether	400,680	470,400	370,080	379,730
Pyridine	90,804		283,870	
Dimethyl Formamide		76,242		120,099

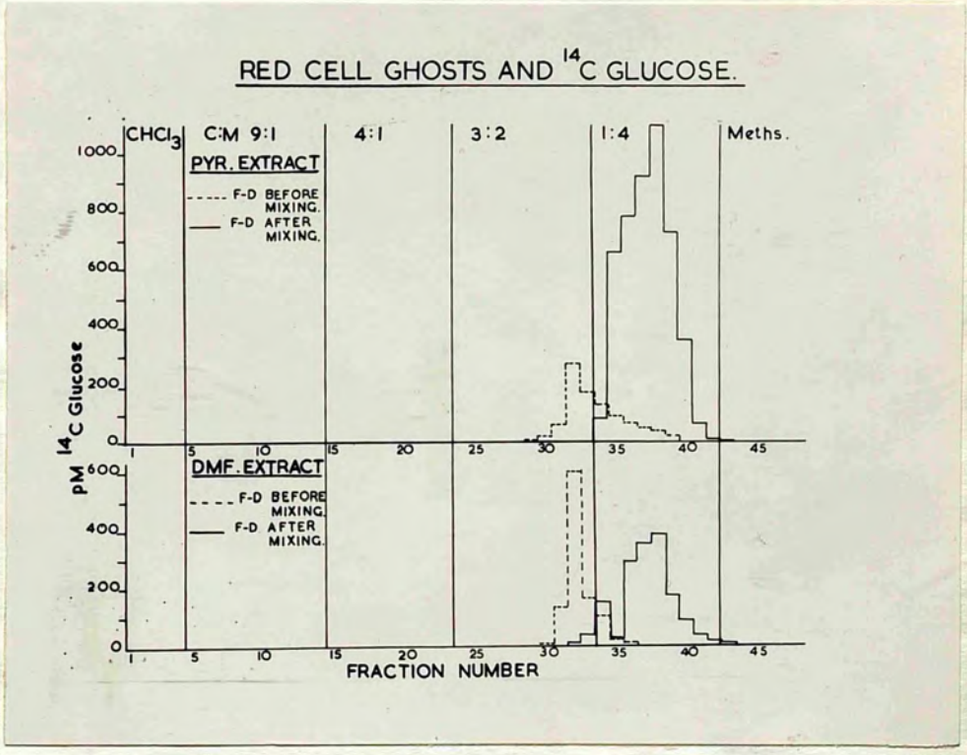
The pyridine fractions of A_1 and B_1 and the dimethyl formamide fractions of A_2 and B_2 were evaporated to dryness under reduced pressure in a nitrogen atmosphere. The residues were redissolved in redistilled chloroform for silicic acid/hyflo column chromatography and all the residue in each case was soluble in the chloroform. The distribution of radioactivity in the eluent fractions of each column is represented on Graph 20.

The pyridine and dimethyl formamide extracts of the control experiment (A_1 and A_2) gave only one region of radioactivity in the 3:2 eluent, corresponding to the elution of free glucose. The pyridine and dimethyl formamide extracts of the standard experiment (B_1 and B_2) gave radioactivity elution only in the 1:4 eluent, which corresponded to the original region from which the complex material had been obtained.

Samples of all four columns were selected for thin-layer chromatography.

Column:			
	Pyridine A	(A_1)	Fractions 35 & 36
	Pyridine B	(B_1)	Fractions 38
	DMF A	(A_2)	Fractions 34 & 35
	DMF B	(B_2)	Fractions 39

The plate was run in a dimethyl formamide solvent and the radioactive scans of all four samples are represented on Fig.19. Both fractions from the control columns



Graph 20. Silicic acid/Hyflo columns of the pyridine soluble material of Experiments A_1 and B_1 and of the dimethyl formamide soluble material of Experiments A_2 and B_2. The radioactive glucose per fraction is represented.

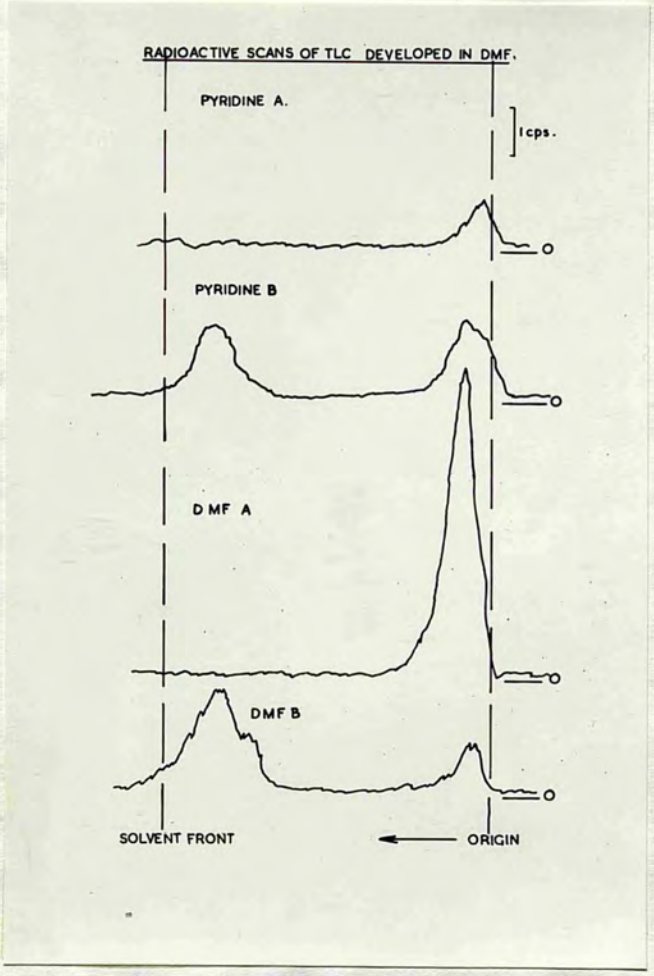


Fig.19. Radioactive scans of material from the C:M, 1:4 column eluent of the pyridine soluble material of experiments A_1 and B_1 and of the dimethyl formamide soluble material of experiments A_2 and B_2. The plate was chromatographed in dimethyl formamide.

(A_1 and A_2) gave only one peak of radioactivity at the origin, corresponding to free glucose. The fractions from the standard experiments (B_1 and B_2) gave a second peak at Rf 0.85. Iodine and ninhydrin detection staining of the plate was unsuccessful, but a spot of Rf value 0.85 in the pyridine and dimethyl formamide fractions of experiment B fluoresced under ultraviolet light.

Similarities with Component of Triphosphoinositide Sample

Material with the same chromatographic properties to that described above was obtained when a sample of triphosphoinositide, prepared from rat brains, was incubated with a ^{14}C glucose solution in pyridine. Thin layer chromatography of the plates developed in a dimethyl formamide solvent system showed the material to have two radioactive components, one of Rf value 0.05, corresponding to free glucose, and a second of Rf value 0.85. The latter fluoresced under U.V. light as had the material from red cell ghosts.

Although the dimethyl formamide effected separation of the radioactivity into two components, this solvent limited subsequent chemical identification of the components separated on the plate. The behaviour of the triphosphoinositide preparation in a number of solvent systems was studied. Chloroform-methanol systems of different solvent ratios were used in tanks for the small chromatography plates. The chromatographed plates could then be charred with sulphuric acid. Fig.20 shows a triphosphoinositide sample run with other lipid standards in C-M 1:1 (v/v), 1:2 (v/v) and 1:3 (v/v) systems. The chromatographed plates were developed by saturation of the lipids in an iodine tank. Fig.21 shows the same plates after charring with sulphuric acid. Both methods of lipid development suggested that the C-M 1:2 solvent system gave the best lipid separation without excessive streaking of the lipids.

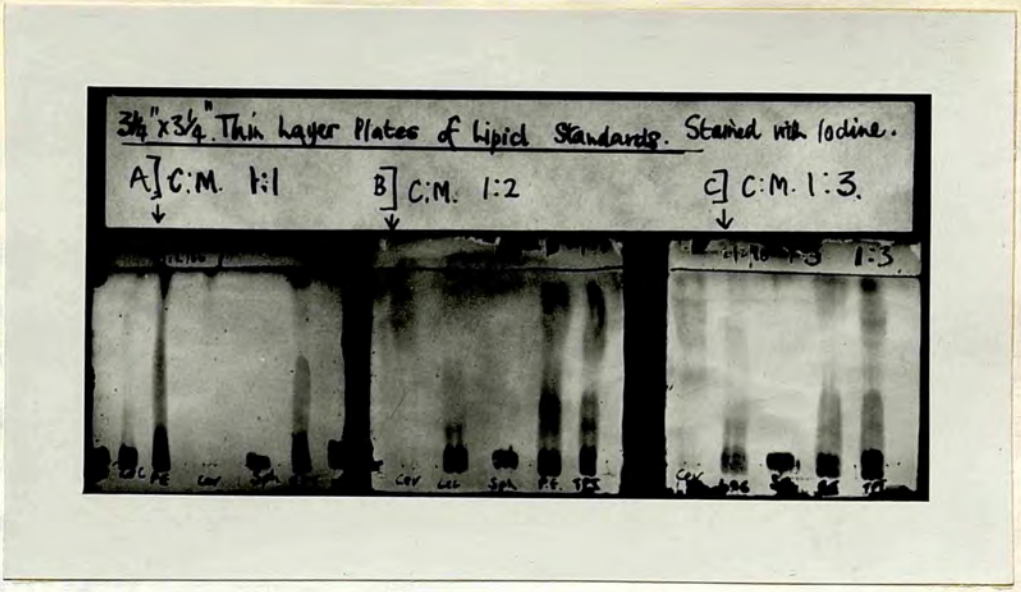


Fig.20. Thin layer chromatogram of a triphosphoinositide preparation and of lipid standards chromatographed in (A) C:M 1:1, (B) C:M 1:2 and (C) C:M 1:3. The plates were developed in a saturated iodine tank.

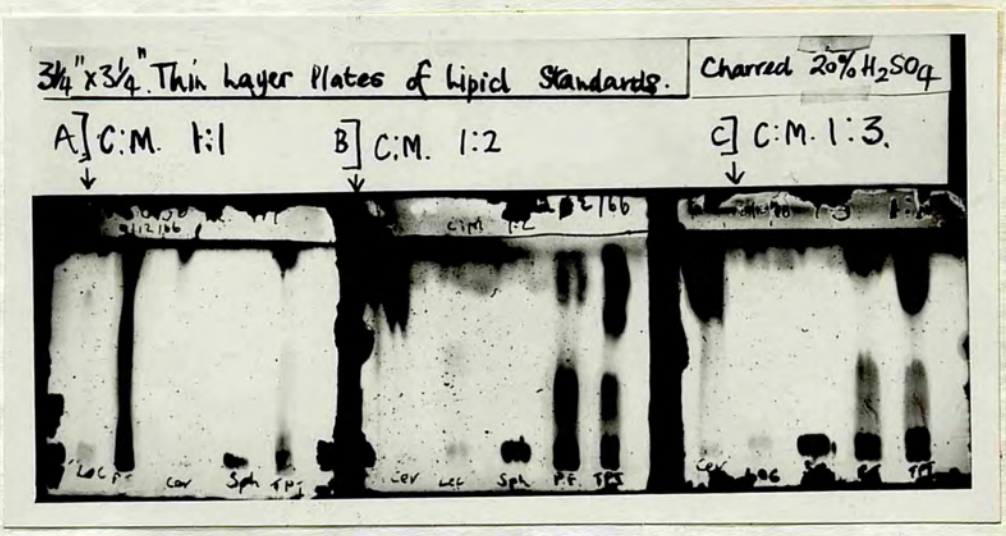


Fig.21. As Fig.20 after charring with 20% sulphuric acid.

During the charring of the triphosphoinositide preparation, several colour changes were observed. Most noticeable were (a) an area travelling with the solvent front which was initially red, then dark brown and finally black, and (b) a spot travelling just behind it which was initially blue, then dark grey and finally black. The former region was also present in some of the other lipid standards and was probably neutral lipids, but the latter spot was peculiar to the triphosphoinositide sample.

Chromatography of a triphosphoinositide preparation which had been incubated with ^{14}C glucose showed that some of the radioactivity was present in the region of the blue material. Fraction 38, from a silicic acid column of a pyridine extract of a previous experiment, gave a similar result to that of the radioactive triphosphoinositide preparation (Fig.22).

A purer sample of triphosphoinositide (donated by Dr. Dawson) was chromatographed in the three solvent systems C-M-H_2O 80:30:3, C-M 1:2 and DMF. All plates were charred with sulphuric acid and Figs. 23 and 24 show the developed chromatographs. The results showed the material previously used to have been of a similar degree of purity to the donated triphosphoinositide. Neither sample gave a clear positive phosphate stain with the Dittmer reagent, although negative results were observed with most of the commercial lipid standards.

The C-M-H_2O solvent system gave the best separation of the components of the triphosphoinositol preparation but failed to give any separation of the components of a radioactive preparation, all the radioactivity remaining at the solvent front.

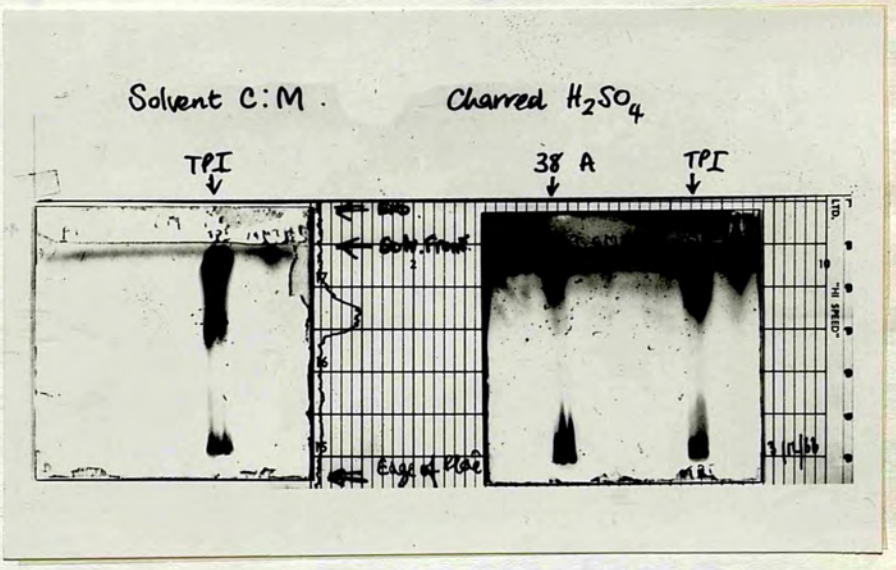


Fig.22. Radioactive scan of the triphosphoinositide + ^{14}C glucose preparation and of Fraction 38 of a silicic acid column of a pyridine extract. The plate was chromatographed in C:M 1:2 and developed by charring with 20% sulphuric acid.

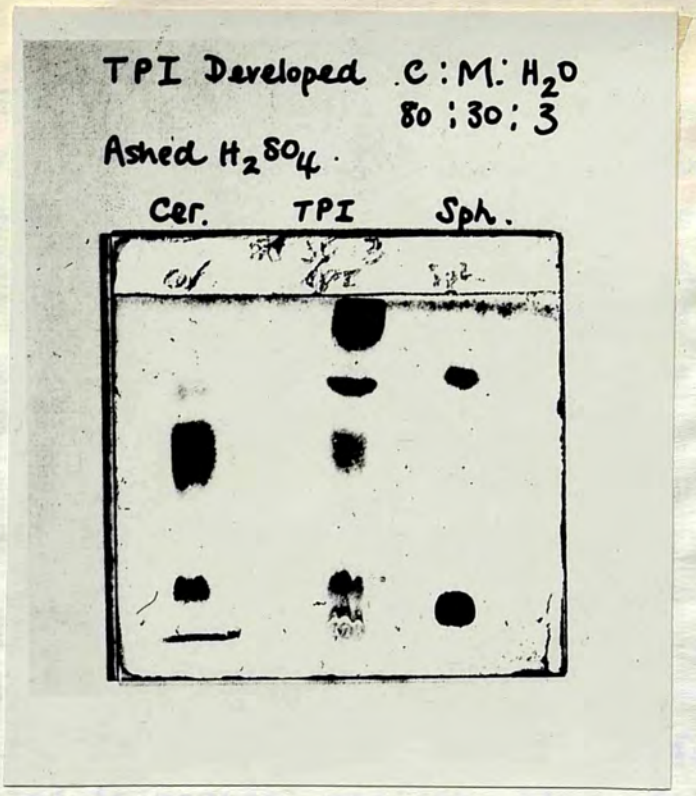


Fig.23. Thin layer chromatogram of a sample of triphosphoinositide donated by Dr. R.M.C. Dawson. The plate was chromatographed in C:M:H_2O, 80:30:3 and developed by charring with 20% sulphuric acid.

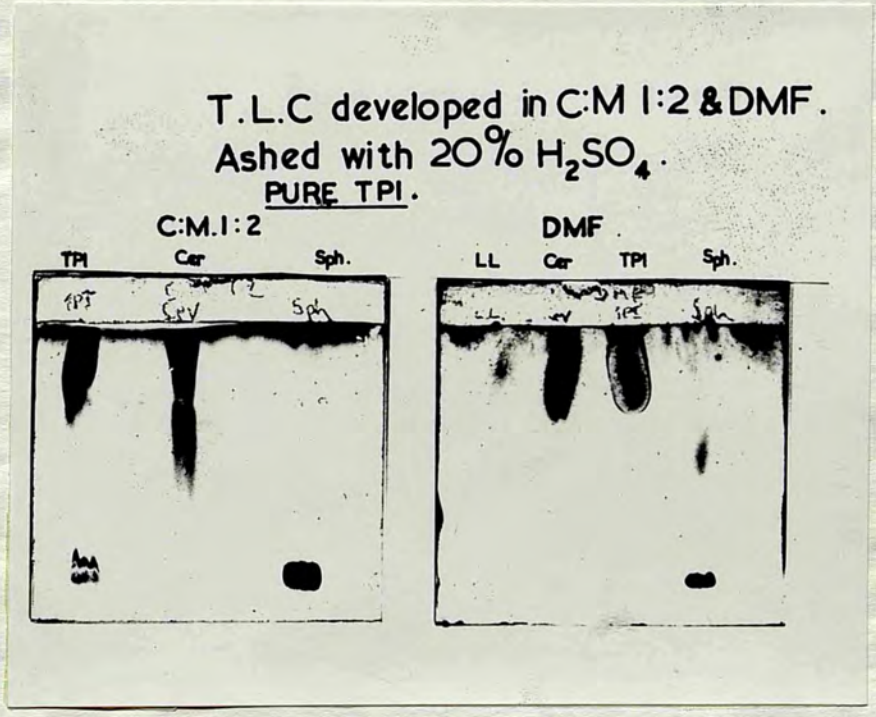


Fig.24. As Fig.23 but the plates were chromatographed in C:M 1:2 and dimethyl formamide solvent systems and then developed by charring with 20% sulphuric acid.

According to Handa et al (1964) the pyridine extract of the methanol-ether residue should contain the globosides of the red cell and it seemed possible that the method used for the extraction of triphosphoinositide from the rat brains also extracted the glycolipids from the brain tissue. Silicic acid/hyflo column chromatography was performed on a triphosphoinositide preparation and one set of alternate fractions were chromatographed on thin-layer plates in a C:M 1:2 solvent system, while the others were used for the estimation of glycolipid by the anthrone method. The crude triphosphoinositide preparation was also applied to the plates. Fig.25 shows the plates after sulphuric acid charring. Material corresponding to that previously observed at R_f 0.8 and a blue colour was eluted in several regions, viz., (i) fractions 21-23, (ii) fractions 31-33 and (iii) fractions 37-41. This closely agrees with that recorded by Yamkawa et al for the elution of human erythrocyte M-E glycolipid using a similar series of chloroform-methanol eluents. Carbohydrate estimations performed on some of the remaining alternate fractions using the method described, the same authors gave the following results :-

Fractions	Optical Density	Total Sugar	Av. µg. Sugar/ Fraction
12,14,18	1.4	270 µg	90
20,22,24	0.34	66 µg	22
30,32	0.137	26 µg	13
36,38	0.194	27.4 µg	18.7
44,46	0.081	15.6 µg	7.8

The high sugar content of Fractions 12,14 and 18 was accounted for by the finding that cerebroside was eluted in this region when a mixture of lipid standards was applied to a silicic acid/hyflo column and eluted with the same solvents. The small peak of sugar present in the 36 and 38 fractions corresponded to the region of radioactive elution of the pyridine extract of the methanol-ether lipid residue. The

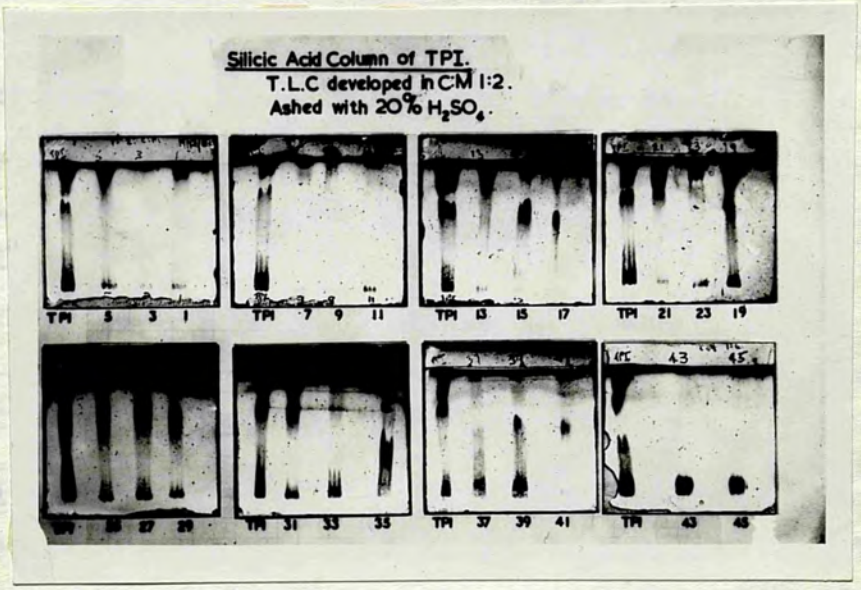


Fig.25. Thin layer chromatograms of the eluent fractions of the silicic acid column of the triphosphoinositide preparation. The plates were chromatographed in C:M 1:2 and developed by charring with 20% sulphuric acid.

finding of these zones of glycolipids make it possible that the component in the triphosphoinositide sample which complexes glucose in pyridine is also a contaminant extracted along with triphosphoinositide from rat brain.

Sugar estimations were carried out on the eluent fractions of a pyridine extract of red cell lipids. In the experiment, the quantity of radioactive sugar added to the red cell ghosts was of the order of 6 µg. and no ^{12}C glucose was added to keep the level well below detection by the anthrone reagent method.

The ghosts were freeze-dried before lipid extraction and four phials were used for the experiment, each containing :-

1.7 ml. ghosts
0.2 ml. radioactive sugar
0.1 ml. saline

The solutions in the phials were well mixed and incubated for one minute at ice-cold temperatures. When completely dry, the powdered ghosts were extracted with methanol-ether 1:1 v/v. The residue after methanol-ether evaporation was successively extracted with acetone, diethylether and pyridine solvents. The distribution of radioactivity in each extract was :-

Solvent	Total Radioactivity
M-E	1,050,090 counts
Acetone	349,200 "
Diethylether	280,440 "
Pyridine	274,500 "

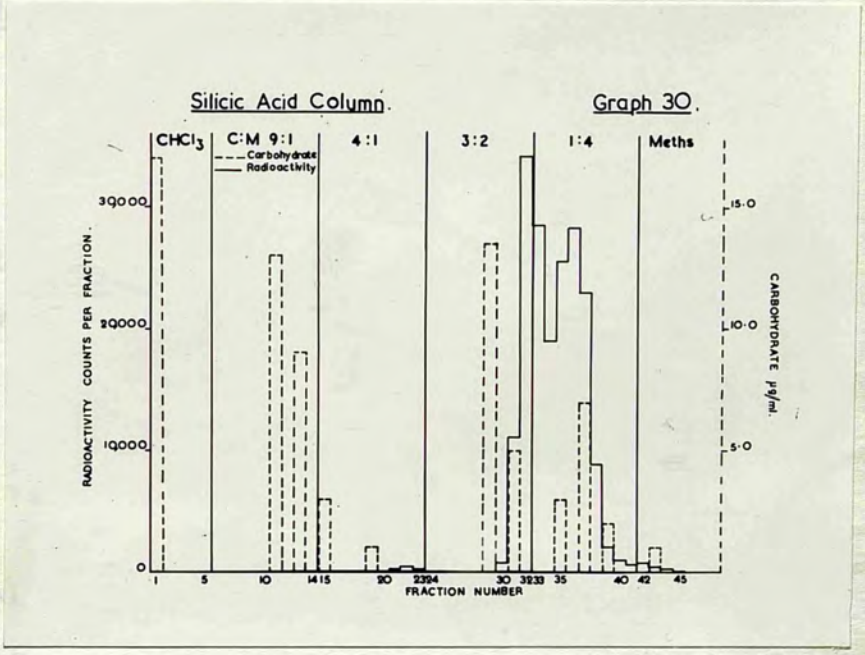
The pyridine extract was evaporated to dryness under reduced pressure in a nitrogen atmosphere and the residue was dissolved in redistilled chloroform and applied to a silicic acid/hyflo column. Radioactive counts were taken of all eluent fractions while sugar estimations were made on alternate fractions.

Graph 30 represents the results :-

The radioactivity was eluted in two regions (a) fractions 31-34, (b) fractions 35-39. The former region corresponded to the elution of free glucose.

Carbohydrate was present in several regions the most significant being that present in fractions 35-39 i.e., corresponding to the later elution peak of radioactivity. Sialic acid was estimated in the remaining alternate fractions which had not been used for carbohydrate estimations. The colour reagent was the Bial's orcinol solution. None of the fractions gave a positive result. Thus the lipid eluted in fractions 35-39 has carbohydrate in its molecular structure similar to that of the structure of the glycolipid, globoside I, of Handa. Radioactivity was associated with this material but further identification using e.g., thin layer plate chromatography was not possible.

Yamakawa et al found that globoside I was eluted in two regions from their silicic acid columns viz. (i) in the C:M 3:2 elution solvent and (ii) in the C:M 2:3 solvent. Thus it is possible, if glycolipid can complex glucose, that not all the radioactivity previously eluted in fractions 31-34 was of necessity free glucose. It has been observed on several occasions that thin layer chromotography of this material in dimethylformamide gave a small quantity of material of R_f value 0.85 but if there is a glycolipid which can complex glucose present in this region it must be extracted in ether and not pyridine when the Handa method is used.



Graph 30. Silicic acid/Hyflo column of the pyridine soluble material of a methanol:ether 1:1 extract of a freeze-dried preparation of ^{14}C glucose and ghosts. The radioactive counts per fraction are represented by the continuous line graph and carbohydrate per alternate fraction by the broken line graph.

b. The effects of increased sugar concentration in the incubation media.

In the earlier experiments butanol and acid-butanol extractions were performed on a series of equal quantities of ghosts incubated in media of increasing sugar concentration. The period of incubation was for one minute and all solutions were at ice-cold temperatures. The experiment was set up as follows :-

Expt.	Ghosts	^{14}C Glucose	^{12}C Glucose	0.9% Saline
A	50.0 ml.	5.0 ml.	-	5.0 ml.
B	50.0 ml.	5.0 ml.	0.5 ml.	4.5 ml.
C	50.0 ml.	5.0 ml.	5.0 ml.	-

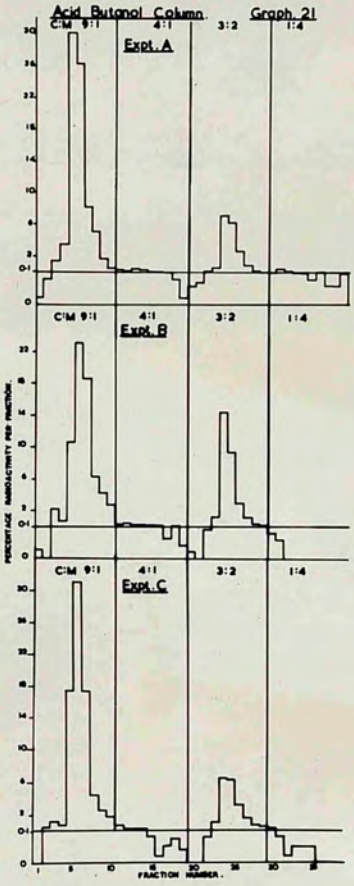
The concentration of the ^{12}C glucose solution was 126 mgm/10 ml. so that the quantity of sugar in each experiment was :-

A - 41 n moles
B - 5.29 μ moles
C - 525 μ moles

The recovery of radioactivity in the extracts of each experiment was :-

Expt.	Stock ^{14}C added	^{14}C in Butanol	^{14}C in Acid-Butanol
A	820,200 counts	275,200 counts	191,000 counts
B	"	315,000 "	223,000 "
C	"	253,000 "	156,000 "

The acid-butanol extracts were evaporated to dryness under reduced pressure in a nitrogen atmosphere. The residues were prepared for silicic acid/Hyflo column chromatography and the radioactivity distribution in the eluent fractions of each column is represented on Graph 21. In all three columns there was a large peak of radioactivity in fractions 3-11 and fraction 6 of this region was strongly coloured with haem pigment.



Graph 21. Silicic acid/Hyflo columns of acid:butanol extracts of aqueous preparations of ghosts incubated with :-

A. 41 n moles of glucose.
B. 5,290 n moles of glucose.
C. 52,500 n moles of glucose.

The percentage recovery of radioactivity per fraction is represented.

The concentration of sugar in the peak material

Column	Fractions	Total Counts	Concentration Glucose
A	4-10	147,320	5.0 n moles
B	3-10	78,000	630 n moles
C	3-11	126,090	81.33 μ moles

To estimate the degree of saturation of the carriers

Assuming that the volume of water in the incubation media was of the order of 40.0 ml., then the approximate concentrations of glucose in the media were calculated.

In experiment A there were 41 n moles of glucose in 40 ml. of water which gave a sugar concentration of 1.02 μ moles/litre. Similarly, in experiments B and C the glucose concentrations were 132 μ moles/litre and 1,313 μ moles/litre. If carriers postulated to be responsible for glucose transfer in intact cells were involved in these extract fractions, the equation

$$\Theta = \frac{C}{C + \phi} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where C = sugar concentration and ϕ = half saturation concentration, would give the actual degree of saturation of the carriers. ϕ varies according to the temperature of the experiment (Sen & Widdas 1962). At room temperature, which is approximately 19-22°C, ϕ is of the order of 1 mM, while at 7°C ϕ is reduced to 0.58 mM. At the temperature of the experiment, which was approaching 0°C, the probable range of ϕ was $0.5 > \phi > 0.2$ mM. 0.3 mM was taken as an arbitrary value of ϕ for this experiment.

For experiment A, $\Theta = \frac{1.02}{1.02 + 300} = 0.00339$

For experiment B, $\Theta = \frac{132}{132 + 300} = 0.3055$

For experiment C, $\Theta = \frac{1313}{1313 + 300} = 0.82$

In experiment B the carrier system was one-third saturated by the sugar associated with the peak material. This quantity of sugar was 630 n moles, which suggested that a fully saturated system for 50.00 ml. of ghosts would be associated with 2,100 n moles of glucose.

In experiment C the carrier system was 0.82 saturated by the sugar associated with the peak material. The quantity of peak sugar was 81,330 n moles and, therefore, on these results, the fully saturated carrier system would be associated with 99,180 n moles of sugar. Thus the material related to the radioactivity in fractions 3-11 did not appear to follow saturation kinetics.

Confirmation of these results was obtained from a similar experiment which was set up as follows :-

Expt.	Ghosts	^{14}C Glucose	^{12}C Glucose	Saline
A	50.0 ml.	5.0 ml.	-	5.0 ml.
B	50.0 ml.	5.0 ml.	0.5 ml.	4.5 ml.
C	50.0 ml.	5.0 ml.	5.0 ml.	-

The concentration of the ^{12}C glucose solution was 189 mgm./10 ml. so that the quantities of glucose in each experiment were :-

A - 163 n moles
B - 5,188 n moles
C - 52,500 n moles

The incubation period of the ghosts with glucose was for one minute at ice-cold temperatures and the butanol was also at ice-cold temperatures. The recovery of radioactivity in the butanol and acid:butanol extracts was :-

Expt.	Stock ^{14}C Added	^{14}C in Butanol	^{14}C in Acid-Butanol
A	1,850,000 counts	575,360 counts	320,625 counts
B	"	620,800 "	398,080 "
C	"	712,470 "	426,600 "

The acid-butanol extracts were evaporated to dryness under reduced pressure in a nitrogen atmosphere and the residues dissolved in redistilled chloroform for application to silicic acid/Hyflo columns. Radioactivity counts were recorded for the eluent fractions and the results are represented in Graph 22. The large peaks of radioactivity in fractions 3-11 were again present in all three columns.

The concentration of sugar in the peak material

Expt.	Fractions	Total Counts	n moles of Glucose
A	3-11	174,980	15.4
B	2-10	333,830	930
C	3-11	194,150	55,100

Assuming the quantity of water in each incubation media to be of the order of 40.0 ml., then the concentration of glucose in each experiment was approximately :-

A - 4.1 μ moles/litre
B - 130 μ moles/litre
C - 1250 μ moles/litre

Thus, using the previous equation (1) and taking ϕ = 3 mM (300 μM), for experiment A

$$\Theta = \frac{4.1}{300 + 4.1} = 0.0135$$

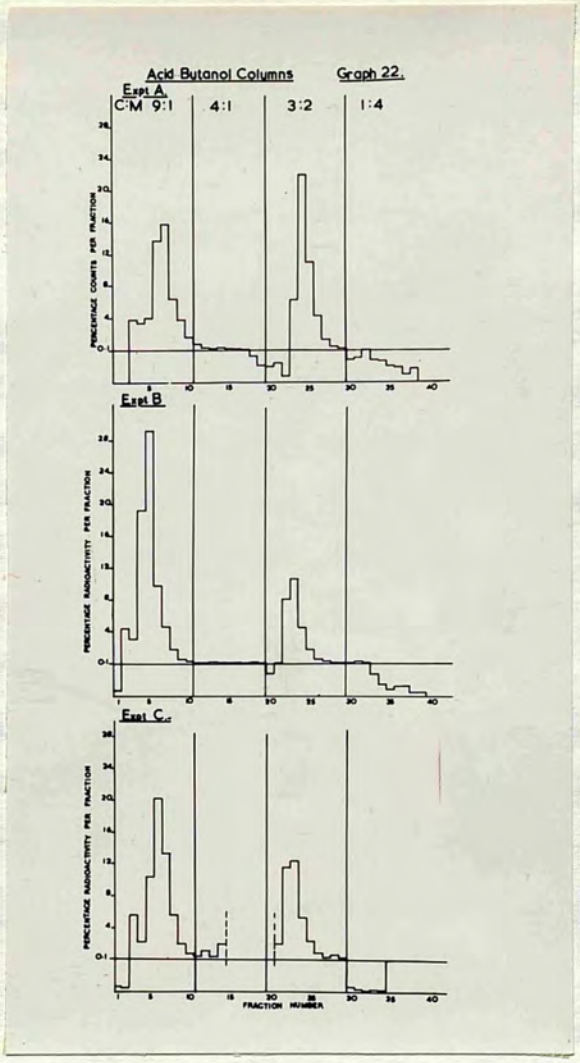
for experiment B

$$\Theta = \frac{130}{300 + 130} = 0.306$$

for experiment C

$$\Theta = \frac{1250}{1250 + 300} = 0.806$$

From Θ of experiment B the carrier system was approximately one-third saturated by the sugar associated with the peak material. This quantity of sugar was 930 n moles and



Graph 22. Silicic acid/Hyflo columns of acid:butanol extracts of aqueous preparations of ghosts incubated with :-

A. 163 n moles of glucose.
B. 5,188 n moles of glucose.
C. 52,500 n moles of glucose.

The percentage recovery of radioactivity per fraction is represented.

therefore approximately 2,846 n moles of glucose would be associated with a fully saturated carrier system for 50.0 ml. of ghosts under the experimental conditions.

From experiment C, θ was 0.806 and the sugar associated with the peak material was 55,100 n moles. From these values the fully saturated carrier system would be associated with 68,362 n moles of glucose.

Since equal quantities of ghosts were used and identical experimental conditions prevailed in experiments B and C, the results did not support saturation kinetics in the formation of the material associated with the peak radioactivity. But it was later found that some of the material eluted in fractions 3-11 was artifact, so that neither of the previous results could be considered valid.

In a later experiment an isopropanol-chloroform 11:7 v/v extraction was performed on a series of incubation media similar to those of the previous experiment.

Expt.	Ghosts	Stock ^{14}C Glucose	Stock ^{12}C Glucose	Saline
A	50.0 ml.	5.0 ml.	-	1.0 ml.
B	50.0 ml.	5.0 ml.	0.1 ml.	0.9 ml.
C	50.0 ml.	5.0 ml.	1.0 ml.	-

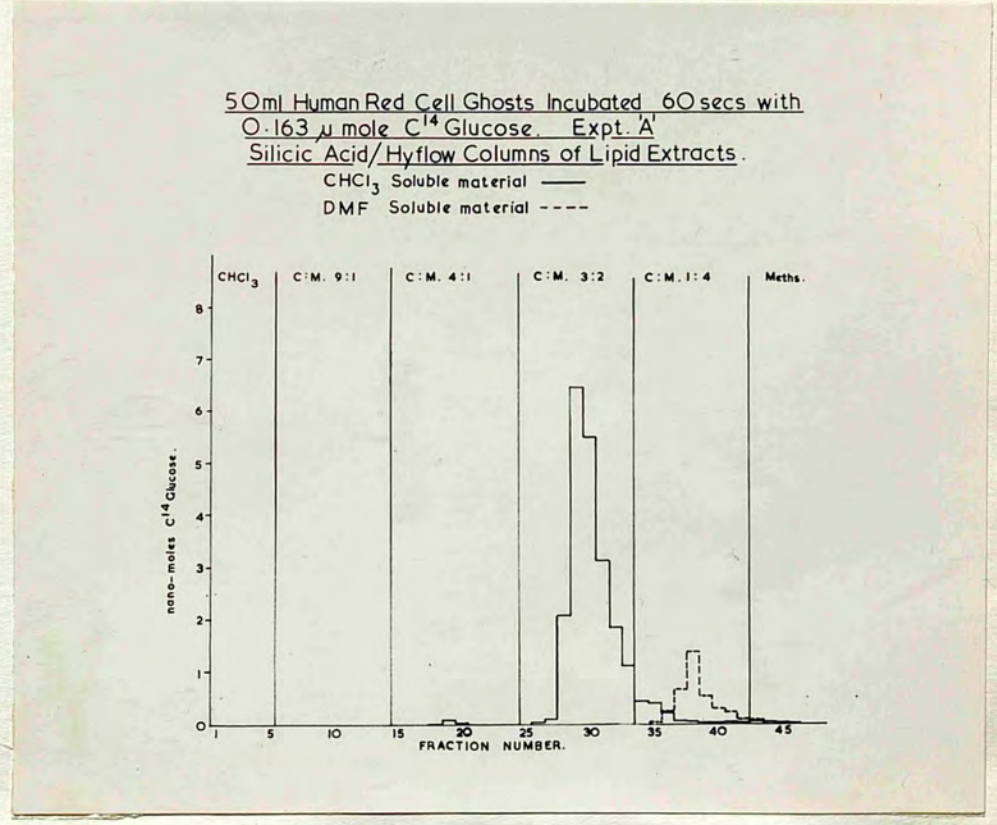
The ghosts and glucose were incubated at ice-cold temperatures for one minute and were then extracted with ice-cold isopropanol-chloroform 11:7 v/v. The concentration of the ^{12}C glucose solution was 189 mgm./10 ml. so that the concentration of glucose in each experiment was :-

A - 0.163 μ moles
B - 10.66 μ moles
C - 105.1 μ moles

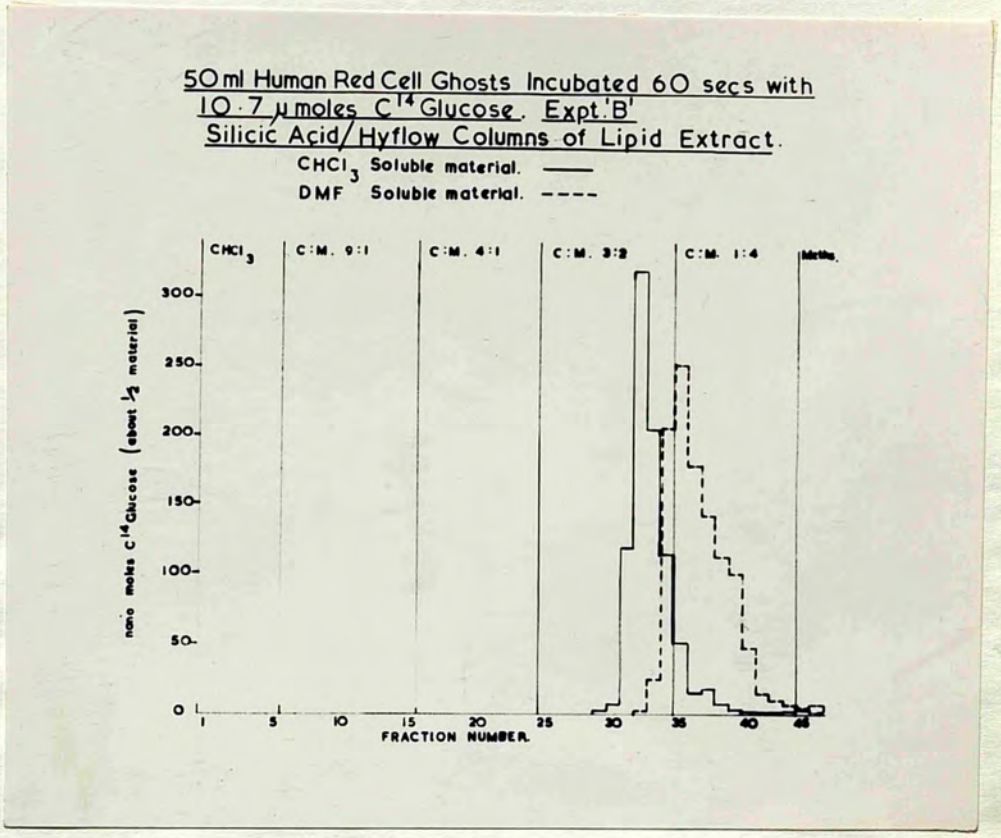
The recovery of counts was :-

Expt.	^{14}C Glucose Added	^{14}C Glucose in Isopropanol/Chloroform
A	1,500,000 counts	1,386,900 counts
B	"	1,348,560 "
C	"	1,205,710 "

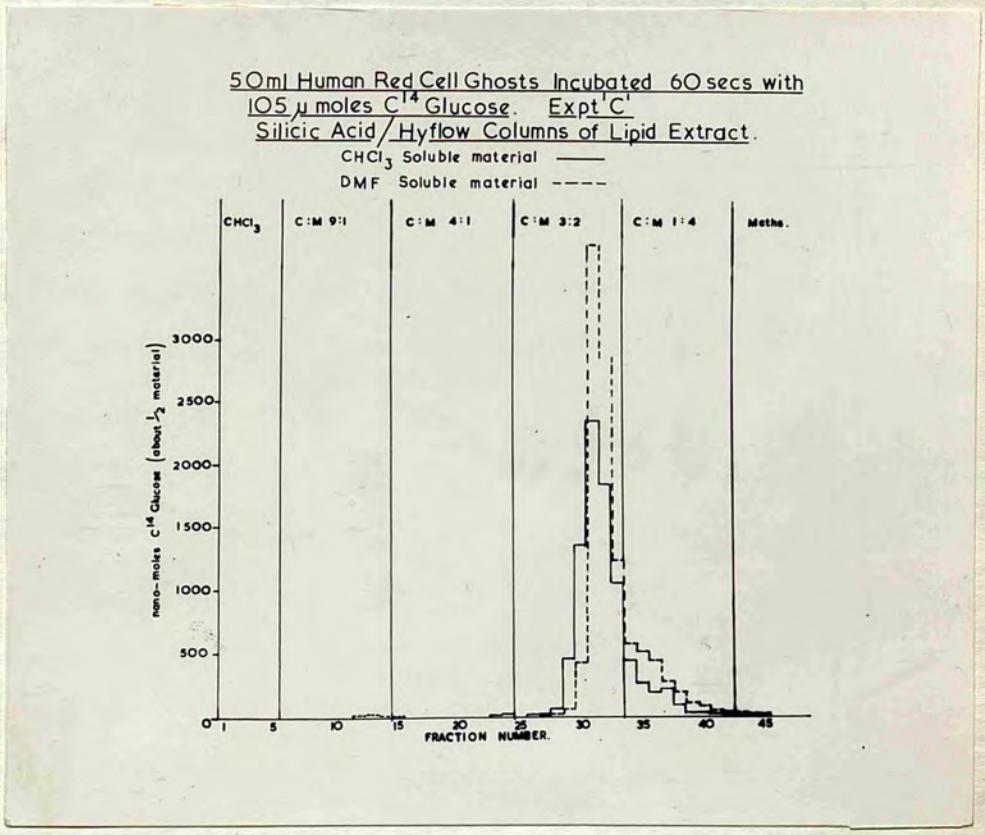
All three isopropanol-chloroform extracts were evaporated to dryness under reduced pressure in a nitrogen atmosphere. The residues were dissolved in redistilled chloroform and applied to silicic acid/Hyflo columns. There was insoluble material in all three chloroform extracts and the chloroform-soluble supernatants were removed using Pasteur pipettes and applied to the silicic acid/Hyflo columns. The insoluble material was re-extracted with redistilled dimethyl formamide. Separate silicic acid/Hyflo columns were run of the three chloroform and dimethyl formamide extracts. Graphs 23, 24 and 25 represent the distribution of radioactivity in the eluent fractions. All three chloroform extracts had large peaks of radioactivity in the C-M 3:2 eluent, corresponding to the radioactivity elution pattern of free glucose. The dimethyl formamide extracts had radioactivity in the C-M 3:2 and in the C-M 1:4 eluents. In experiment A only the 1:4 eluent radio-activity was present. In experiments B and C there were radioactivity peaks in the C-M 3:2 and in the C-M 1:4 solvents. These two elution peaks overlapped and to remove any free sugar from the later peak, fractions 35-45 were pooled, evaporated to dryness and the residue taken up in methanol-diethyl ether, 1:1 v/v. The extract was evaporated to dryness under reduced pressure in a nitrogen atmosphere and the residue was successively extracted with acetone, diethyl ether and pyridine. It had previously been observed that free sugar was extracted into both the acetone and ether solvents. The distribution of counts in each solvent was :-



Graph 23. Silicic acid/Hyflo columns of the chloroform and dimethyl formamide soluble material of an isopropanol:chloroform 11:7 extract of an aqueous preparation of ghosts and 0.163 μ mole of ^{14}C glucose. The radioactive sugar per fraction is represented.



Graph 24. Silicic acid/Hyflo columns of the chloroform and dimethyl formamide soluble material of an isopropanol:chloroform 11:7 extract of an aqueous preparation of ghosts and 10.7 µ moles of ^{14}C and ^{12}C glucose. The radioactive sugar per fraction is represented.



Graph 25. Silicic acid/Hyflo columns of the chloroform and dimethyl formamide soluble material of an isopropanol:chloroform 11:7 extract of an aqueous preparation of ghosts and 105 μ moles of ^{14}C and ^{12}C glucose. The radioactive sugar per fraction is represented.

Expt.	Fractions	Acetone	Ether	Pyridine
B	35-45	8,070 counts	1,350 counts	27,150 counts
C	35-45	5,850 "	410 "	8,975 "

The pyridine extracts were evaporated to dryness under reduced pressure in a nitrogen atmosphere and the residues were dissolved in redistilled chloroform for application to silicic acid/Hyflo columns.

The radioactivity in both extracts chromatographed to give only one region of radioactivity in the C-M 1:4 v/v solvent. It was assumed that the radioactivity in the pyridine extracts may have represented the quantity of sugar associated with the carrier system for the particular experiment.

The quantity of water in the incubation media was assumed to be approximately 35.0 ml. so that the glucose concentration for each experiment was :-

A - 4.89 μ moles/litre
B - 321 μ moles/litre
C - 3150 μ moles/litre

Assuming to be of the order of 0.3 mM and using equation (1),

in experiment A

$$\Theta = \frac{4.89}{4.89 + 300} = 0.0164$$

in experiment B

$$\Theta = \frac{321}{321 + 300} = 0.157$$

in experiment C

$$\Theta = \frac{3150}{3150 + 300} = 0.914$$

In experiments B and C the quantities of sugar associated in the pyridine were 193 and 628 n moles respectively. Using the calculated values of 'ΘB' and 'ΘC', the quantities of sugar which would have been associated with a fully saturated system

for 50.0 ml. of ghosts were 355 n moles and 668 n moles respectively. Thus the result from experiment C was almost twice that from experiment B, so that saturation kinetics do not appear to be followed.

The variation of ϕ with temperature would greatly affect the results. If the value of ϕ was 0.5 and not 0.3 mM, then the quantities of sugar associated with a fully saturated system, calculated from ~~θ~~B and ~~θ~~C, would be of the order of 551 and 730 n moles respectively. Since it was unlikely that radioactive material losses were the same in all three experiments, no final conclusions could be drawn.

Freeze-drying the ghosts before lipid solvent extraction excludes water from the lipid extract and so decreases the possibility of complex dissociation during the last stages of evaporation of the lipid solvent. Three stock ^{12}C glucose solutions were prepared :-

Stock ^{12}C glucose solution experiment B - 14.4 mgm./5.0 ml.
" " " " " C - 50.4 mgm./5.0 ml.
" " " " " D -176.4 mgm./5.0 ml.

In experiment A the stock ^{12}C glucose solution was replaced by 0.9% saline. The experiments were set up :-

Expt.	Phials	^{14}C Glucose	Saline	^{12}C 'B'	^{12}C 'C'	^{12}C 'D'
A	3	0.2 ml.	0.1 ml.	-	-	-
B	3	0.2 ml.	-	0.1 ml.	-	-
C	3	0.2 ml.	-	-	0.1 ml.	-
D	3	0.2 ml.	-	-	-	0.1 ml.

All solutions were at ice-cold temperatures and the phials were cooled before the introduction of the sugar and ghosts. The ghosts and sugar were incubated for one minute with thorough mixing before positioning in the freeze-drying apparatus. When dry, the powdered ghosts were throughly

extracted with methanol-diethyl ether 1:1 v/v and radioactivity counts of the extract recorded. The extract was evaporated to dryness under reduced pressure in a nitrogen atmosphere and the residue successively extracted with acetone-diethyl ether and pyridine. Radioactivity counts were recorded of all extracts.

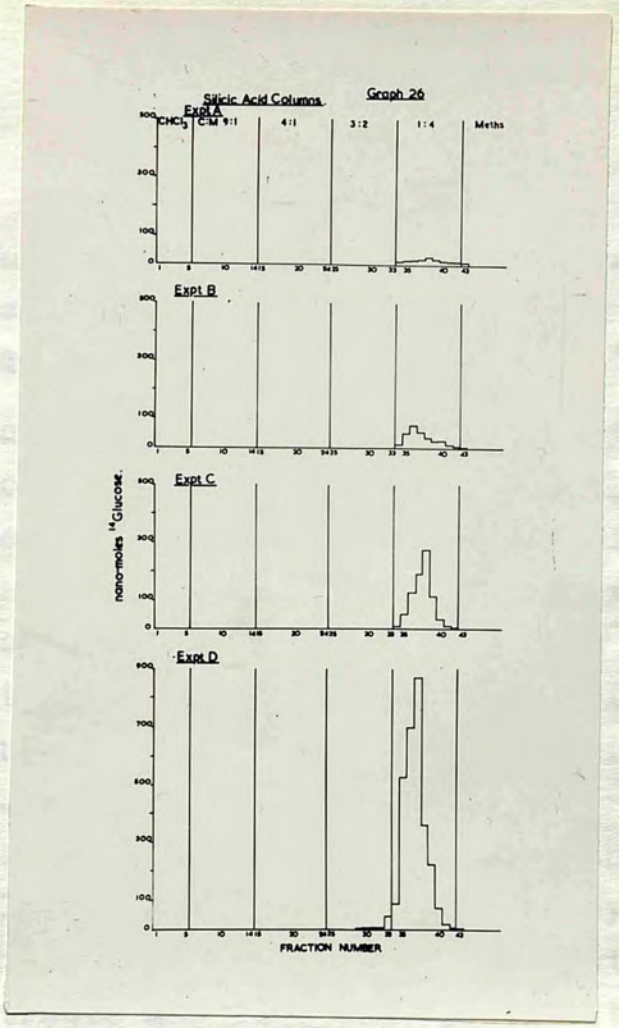
Expt.	^{14}C Added	^{14}C M-E	^{14}C Acetone	^{14}C Ether	^{14}C Pyridine
A	896,250 cts.	401,072cts.	45,170 cts.	22,995 cts.	342,000 cts.
B	"	464,040 "	60,750 "	57,420 "	348,000 "
C	"	509,480 "	36,750 "	90,500 "	285,000 "
D	"	484,100 "	29,768 "	70,200 "	225,000 "

The quantities of sugar in each experiment and the recoveries in the pyridine extracts were :-

Expt.	Total glucose present	n moles glucose in the pyridine extract
A	1.2 μ moles	458
B	6.0 μ moles	2, 332
C	18.0 μ moles	5, 728
D	60.0 μ moles	18, 425

The four pyridine extracts were evaporated to dryness under reduced pressure in a nitrogen atmosphere and the residues were dissolved in redistilled chloroform for application to silicic acid/Hyflo columns. Radioactivity in eluent fractions is represented in Graph 26. (There was no insoluble material in any of the chloroform extracts).

In all four chromatographs there was only one region of radioactivity which was in the C-M 1:4 v/v solvent. Assuming this result to indicate the homogeneity of the pyridine extract, the quantity of sugar in the pyridine was directly related to the degree of saturation in each experiment.



Graph 26. Silicic acid/Hyflo columns of the pyridine soluble material of methanol:ether 1:1 extracts of freeze-dried preparations of :-

A. Ghosts and 1.2 μ moles of ^{14}C glucose.
B. Ghosts and 6.0 μ moles of ^{14}C and ^{12}C glucose.
C. Ghosts and 18 μ moles of ^{14}C and ^{12}C glucose.
D. Ghosts and 60 μ moles of ^{14}C and ^{12}C glucose.

The radioactive sugar per fraction is represented.

The concentrations of glucose in each experiment were :-

Experiment A - 0.2 mM/litre
" B - 1.0 mM/litre
" C - 3.0 mM/litre
" D - 10.0 mM/litre

It will be observed that the recoveries in nano-moles were approximately proportional to the concentration prior to extraction, and there is no evidence of any saturation component in this range.

Although column chromatography of the pyridine extracts indicated the homogeneity of the material, thin-layer chromatograms developed in a dimethyl formamide solvent showed the presence of free glucose in the pyridine extracts of experiments B, C and D (Fig.26). The free sugar might have arisen as the result of the breakdown of complex material during the preparation of the extracts for thin-layer chromatography or during the running of the solvent. At higher glucose concentration there may have been an overspill of free glucose into the pyridine solvent, and it was possible that not all the radioactivity in the pyridine extracts had been associated with lipid material. Nevertheless, the results, as such, did not support a reaction of a saturation type between some lipid component and glucose.

c. The effects of known inhibitors of the transport system on lipid:sugar distribution

Irreversible Inhibitors

i. Dinitrofluorobenzene - DNFB

Inhibition of the sugar transport system of red cells results when the cells are preincubated with DNFB before introduction to the sugar medium.

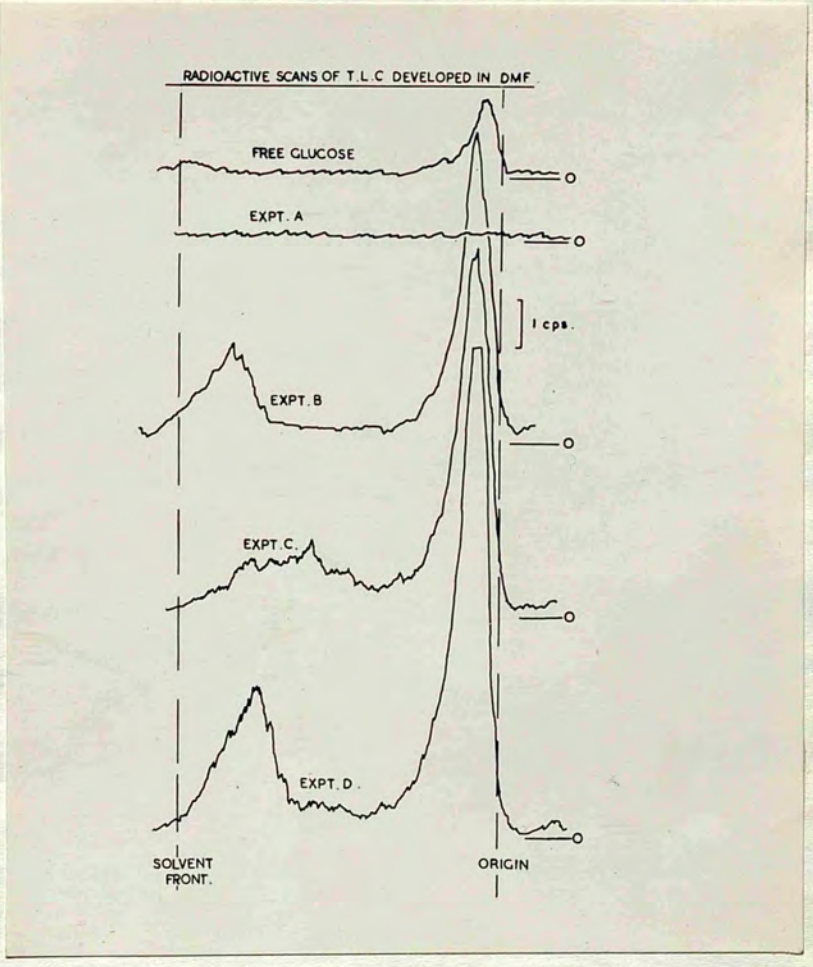


Fig.26. Radioactive scans of the pyridine extract material of experiments A, B, C and D. The plates were chromatographed in dimethyl formamide.

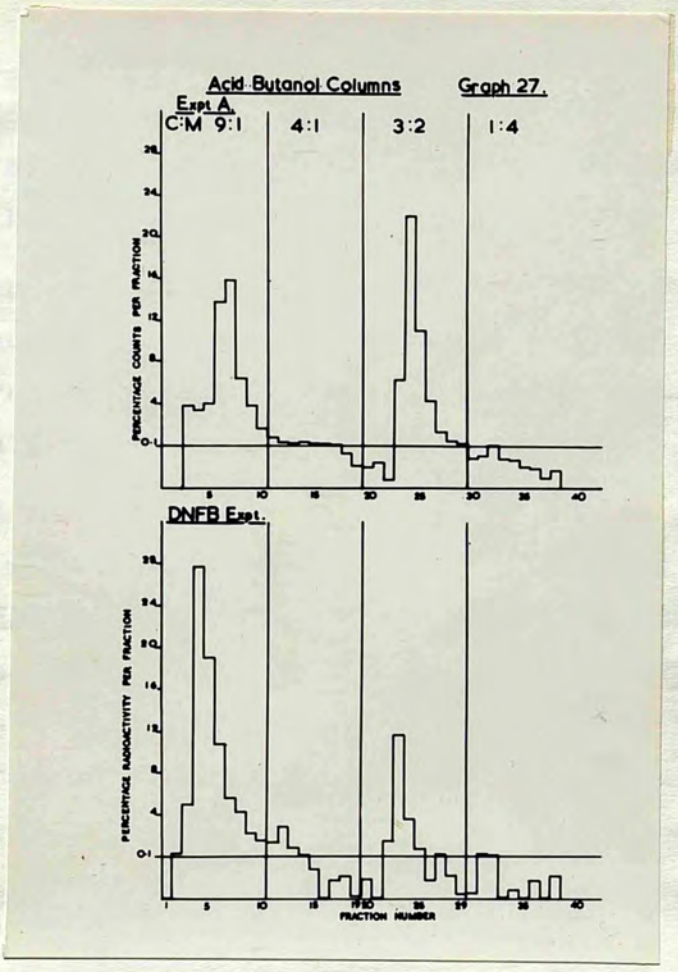
Two 50.0 ml. portions of red cell ghosts were used in an experiment where one portion was preincubated with a DNFB solution. 50.0 ml. of ghosts were incubated with a 5.0 ml. of a DNFB solution 1.0 ml. of which was alcohol and the other 4.0 ml. was 0.9% saline. The final concentration of the DNFB was of the order of 4mMolar. The ghosts and DNFB were incubated for 30 mins. at room temperature before the addition of 5.0 ml. of the stock radioactive glucose solution. The period of incubation of ghosts with sugar in both experiments was one minute with shaking. Successive extractions were performed with ice-cold butanol and ice-cold acid:butanol respectively. The solvent recovery of radioactivity in each experiment was :-

Expt.	^{14}C Stock Glucose	^{14}C Butanol	^{14}C Acid:Butanol
A	719,000 cts/min.	275,000 cts/min.	171,000 cts/min.
B	719,000 "	273,000 "	169,000 "

The concentration of glucose in both experiments was 0.3 μ molar.

At this time the acid:butanol extracts were considered to be of greater interest and consequently only these extracts were prepared for silicic acid/hyflo column chromatography. The acid:butanol was evaporated under reduced pressure and in a nitrogen atmosphere and the dry residue was dissolved in redistilled chloroform and applied to the chromatography column. Graph 27 represents the distribution of radioactivity in the eluent fractions of the two columns. In both columns there were two main regions of radioactivity in the C:M 9:1 and 3:2 eluents. The latter corresponded to the elution peak of free glucose. In both columns fractions 4-6 were contaminated with haem pigment. The quantities of 9:1 peak material were :-

Expt.	Fractions	Total Counts	μmoles Glucose
A	4-10	147,320	7.366
B	3-13	115,560	5.75



Graph 27. Silicic acid/Hyflo columns of the acid:butanol extracts of :-

A. An aqueous preparation of ^{14}C glucose & ghosts.

B. An aqueous preparation of ^{14}C glucose and dinitro-fluorobenzene inhibited ghosts.

The percentage recovery of radioactivity per fraction is represented.

From these results the percentage inhibition by the DNFB

was $\frac{1.616}{7.36} \times 100 = 22\%$.

Since the concentration of DNFB in the incubation media was of the order of 4.0 mM this percentage inhibition seemed too low. However, the peak material was later found to be heterogenous and these results were not valid.

In a later experiment normal ghosts and DNFB inhibited ghosts were incubated with ^{14}C glucose for one minute at ice temperatures in the phials of the freeze-dry apparatus. The dried ghosts were then extracted with methanol-di-ethyl ether 1:1 v/v.

The DNFB inhibited ghosts were prepared by adding 12.0 m/l of ghosts to 48 m/l of DNFB in 0.9% saline to give a final concentration of 4mM. The ghosts were incubated with the DNFB for one hour at 25°C. and then centrifuged from the solution at 25,000 r.p.m. for 20 minutes.

The two experiments were set up as follows :-

Experiment A -

4 phials each containing :-

1.7 ml. of uninhibited ghosts
0.2 ml. ^{14}C glucose solution
0.1 ml. 0.9% saline

Experiment B -

4 phials each containing :-

1.7 ml. of DNFB inhibited ghosts
0.2 ml. ^{14}C glucose solution
0.1 ml. 0.9% saline

8.9 mgm of ^{12}C glucose were added to the stock ^{14}C glucose solution.

The methanol:ether extracts of the powdered ghosts were evaporated to dryness under reduced pressure and in a nitrogen atmosphere. The residues were successively extracted with acetone, diethyl ether and pyridine. The distribution of radioactivity in each solvent was :-

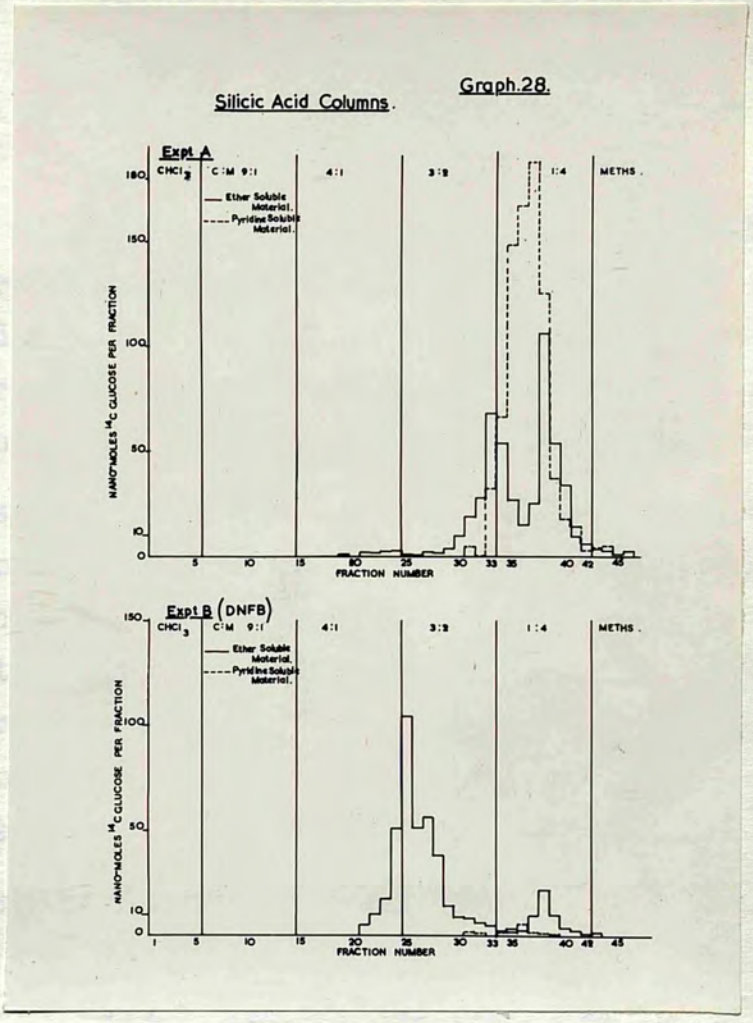
Total Cts/Min.	Experiment A Uninhibited Cells	Experiment B DNFB Treated Cells
M:E	685,400	546,100
Acetone	42,700	77,300
Ether	364,300	486,200
Pyridine	217,700	5,700

The quantity of radioactivity in the pyridine extract of the DNFB treated cells was greatly reduced so that the total sugar present was 0.032μ moles as compared to 1.21μ moles in the untreated cells.

The pyridine and ether extracts of both experiments were evaporated to dryness under reduced pressure and in a nitrogen atmosphere. The residues were dissolved in redistilled chloroform and silicic acid/Hyflo column chromatography performed on each. Graph 28 represents the distribution of sugar in the eluent fractions.

The pyridine extract of experiment A gave two regions of radioactivity one in the 3:2 eluent, in fractions 30-34, and the other in the 1:4 eluent in fractions 36-40. The former corresponded to the elution of free glucose. The ether extract of the same experiment gave one region of radioactivity extending from fractions 32-40 i.e., over the 3:2 and 1:4 eluents.

The pyridine extract of experiment B gave one small region of radioactivity in the fractions 34-39 of the 1:4 eluent. The ether extract gave an entirely different



Graph 28. Silicic acid/Hyflo columns of the pyridine and ether soluble material of methanol:ether extracts of :-

A. A freeze-dried preparation of ^{14}C glucose and ghosts.

B. A freeze-dried preparation of ^{14}C glucose and dinitrofluorobenzene inhibited ghosts.

The radioactive sugar per fraction is represented.

elution pattern from that of any previously recorded, eluting a large peak of radioactivity in fractions 23-29 of the 3:2 eluent. Fractions 18-24 of this column were coloured yellow, the characteristic colour of dinitrophenol and dinitrophenol substituted compounds. A smaller second peak of radioactivity was eluted in fractions 36-40 of the 1:4 eluent.

Fraction 24 and fractions 37, 38 and 39 of the ether column (B) were evaporated to dryness, dissolved in redistilled chloroform and applied to 20 cm x 20 cm silica gel G plates. The plates were chromatographed in a dimethyl formamide solvent and when dry the spots were scanned for radioactivity.

Fig. 27 shows that fraction 25 had a region of radioactivity extending from the origin to Rf value 0.25. Free glucose usually has an Rf value of 0-0.05 in Dimethyl formamide so that the material in fraction 25 appeared to have different migration properties. However, due to the formation of dinitrophenol derivatives there may have been an influence on lipid migration which secondarily affected glucose.

Fractions 37, 38 and 39 (pooled) scanned to give one small peak of radioactivity at Rf value 0-0,1 and a larger peak at Rf value 0.7. The former corresponded to free glucose while the latter peak fluoresced under ultra-violet light and was similar to material previously observed in pyridine extracts at Rf value 0.8-0.85.

Thus the DNFB had not completely inhibited the formation of the sugar-lipid material but had greatly reduced it and had in some way affected the elution of radioactivity in the C-M 3:2 solvent.

Mercuric ions as an inhibitor

A third experiment was performed, in conjunction with the previous two, in which mercuric ions were incubated with the ghosts and radioactive sugar. Four phials were used

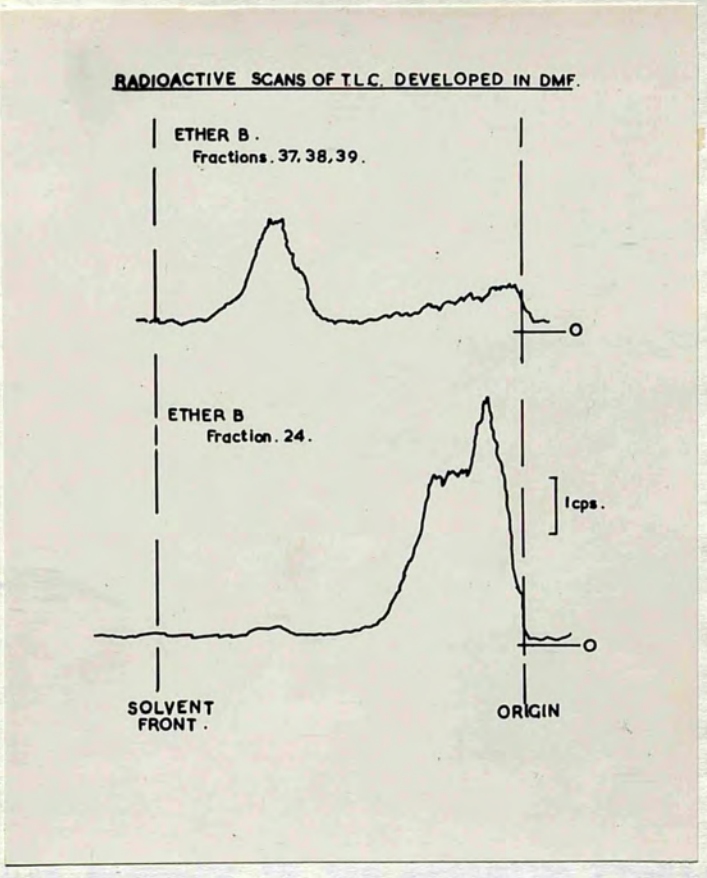


Fig.27. Radioactive scans of Fractions 37, 38 and 39 and of Fraction 25 of the silicic acid column of the ether soluble material of Experiment B. The plate was chromatographed in dimethyl formamide.

for the experiment and were set up as follows :-

1.7 ml. ghosts
0.2 ml. ^{14}C glucose
0.1 ml. mercuric chloride solution

The mercuric chloride solution was 2×10^{-4} Molar. The incubation with ^{14}C glucose was for one minute at ice-cold temperatures and with thorough mixing.

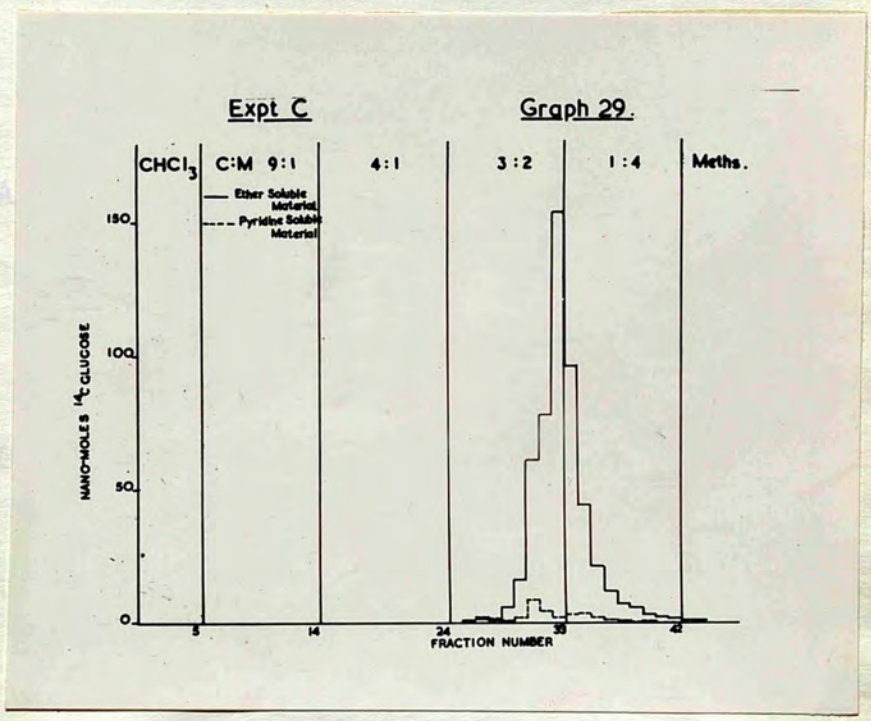
When dry, the powdered ghosts were extracted with M-E 1:1 v/v. This extract was evaporated to dryness under reduced pressure and in a nitrogen atmosphere. The residue was successively extracted with acetone, diethyl ether and pyridine. The distribution of radioactivity in each solvent was :-

Solvent	Total Counts/Min.
M-E	530,100
Acetone	79,300
Ether	458,800
Pyridine	1,400

The total counts present in the pyridine fraction represented only 0.008μ moles of glucose.

The pyridine and ether fractions were evaporated to dryness and the residues were dissolved in redistilled chloroform for application to silicic acid/Hyflo columns. Graph 29 represents the distribution of sugar in the eluent fractions. In the pyridine column the radioactivity was eluted in the fractions 30-33 of the 3:2 C-M eluent. In the ether column the radioactivity was eluted in fractions 29-35 which also consist mainly of the C-M 3:2 eluent. The solvent change from 3:2 to 1:4 C-M ratio occurred at fraction 33.

Neither of these columns showed the radioactive region in the fractions 36-39 of the C-M 1:4 eluent.



Graph 29. Silicic acid/Hyflo columns of the pyridine and ether soluble material of a methanol:ether 1:1 extract of a freeze-dried preparation of ^{14}C glucose and ghosts inhibited with mercuric ions. The radioactive sugar per fraction is represented.

The radioactivity was eluted in the region of free glucose in both columns.

To investigate the mercury inhibition further, use was made of the radioactive mercuric chloride solution available from the Radiochemical Centre, Amersham. In a preliminary experiment, the extraction and chromatographic behaviour of the mercuric ions after incubation with glucose and red cell ghosts was recorded.

In the experiment three equal quantities of ghosts were used. Four freeze-dried phials were used for each preparation and they were set up as follows :-

Experiment A - 4 phials each containing :-
- 1.7 ml. ghosts
- 0.2 ml. stock ^{14}C glucose
- 0.1 ml. 0.9% saline (w/v)

Experiment B - 4 phials each containing :-
- 1.7 ml. ghosts
- 0.2 ml. stock ^{14}C glucose
- 0.1 ml. non-radioactive mercuric chloride solution (concentration 2×10^{-4} M)

Experiment C - 4 phials each containing :-
- 1.7 ml. ghosts
- 0.2 ml. ^{12}C glucose
- 0.1 ml. radioactive mercuric chloride solution (2×10^{-4} M)

The stock ^{14}C glucose solution had a concentration of approximately 4 μg/ml. so that the total radioactive sugar in experiments A and B was of the order of 3.2 μgms. The ^{12}C glucose of experiment C was made up to give the same order of sugar concentration. Thus in all three experiments, the quantity of incubation sugar was well

below the level necessary for detection by the anthrone reagent (10μg.).

Both mercuric chloride solutions were 2×10^{-4} M. All solutions were thoroughly mixed in the phials at 0-4°C and incubated for one minute. The freeze-dried ghosts were extracted with methanol-diethyl ether 1:1 v/v and the radioactivity of the extract recorded.

The M-E extract of experiment C, i.e., the experiment containing the radioactive mercury, had to be filtered a second time after an initial filtration on a Buchner funnel at the pump. The filter paper from the second filtration was highly radioactive and was re-extracted with C-M 1:1.

The M-E extracts of all three experiments were evaporated to dryness under reduced pressure and in a nitrogen atmosphere. The residues were successively extracted with acetone, ether and pyridine. The radioactivity of each extract was recorded.

Expt.	M-E	Acetone	Ether	Pyridine
A	1,050,090	349,200	280,440	274,500
B	1,093,744	515,640	366,120	4,000
C	7,560	1,200	746	5,450

In experiment B the material normally extracted in the pyridine solvent was scarcely, if at all present, while in experiment C very little of the radioactive mercury was recovered in the M-E extract, but 80% of that which was recovered, was extracted in the pyridine solvent.

The pyridine extracts of experiments A and C were evaporated to dryness, and the ether extract of experiment B was similarly evaporated, under reduced pressure and in a nitrogen atmosphere. All three residues were dissolved in redistilled chloroform and applied to silicic acid/Hyflo columns. Radioactive counts were taken of all fractions of pyridine A, while carbohydrate estimations, using the

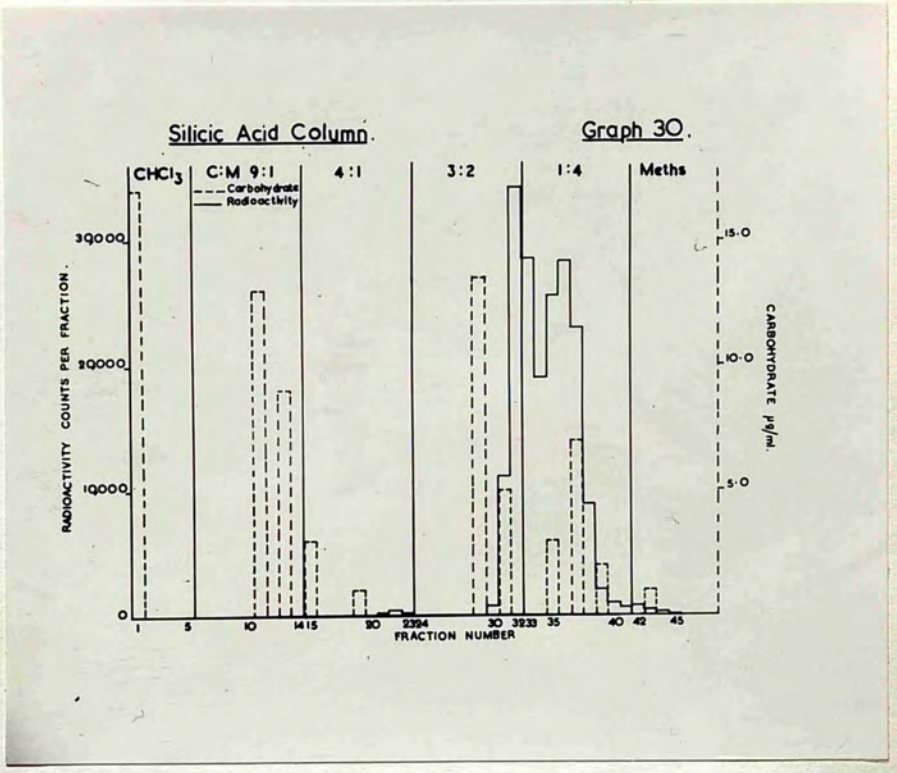
anthrone reagent, were recorded on alternate fractions. The results are represented in Graph 30. There were two main regions of radioactivity : (a) fractions 31-34, (b) fractions 35-39. The former corresponded to the elution pattern of free glucose.

Carbohydrate was present in several regions, the most significant being that in fractions 35-39, thus indicating the possibility of a carbohydrate moiety involved in the structure of the lipid associated with the radioactive sugar in these fractions.

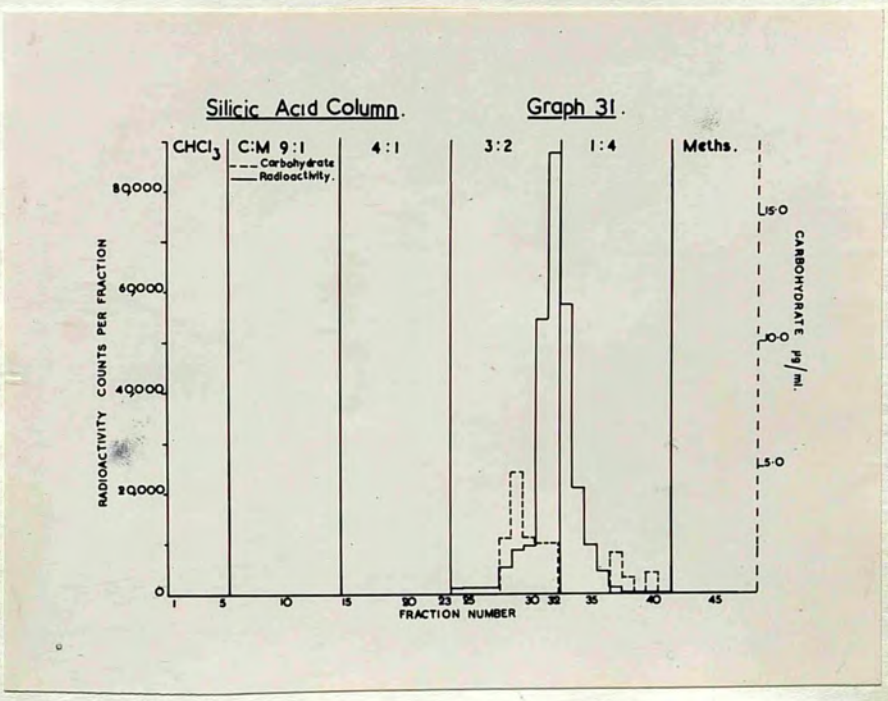
Radioactive counts and carbohydrate estimations were performed on all the eluent fractions of the silicic acid/Hyflo column of the ether B extract, the results are represented in Graph 31. There was one main peak of radioactivity in fractions 29-35, i.e., corresponding to the elution of free glucose. There were two very small regions of carbohydrate in fractions 28-32 and fractions 37-39.

In experiment C radioactive counts and carbohydrate estimations were recorded on all the eluent fractions of the silicic acid/Hyflo column of the pyridine extract. The results are represented in Graph 32. The radioactivity was eluted in a single region, i.e. fractions 35-41. The carbohydrate elution pattern was similar to that of the pyridine extract of experiment A, Graph 30, except that the large quantity of carbohydrate eluted in fractions 11-15 of the latter was now absent and a large quantity of carbohydrate was now present in fractions 19 and 21. The most significant carbohydrate was that present in fractions 35-40 which corresponded to the elution of the radioactive mercury. This suggested that the mercuric ion could have been associated with the "carbohydrate-lipid" which had previously been associated with the radioactive glucose of an uninhibited preparation.

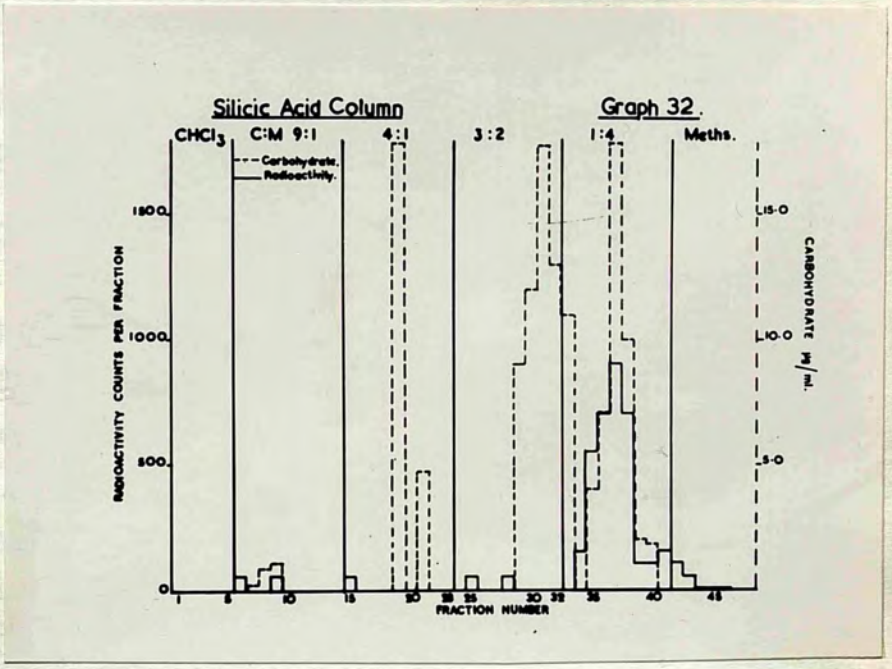
Fractions 36-38 were prepared for thin-layer chromatography on small plates which were developed in three



Graph 30. Silicic acid/Hyflo column of the pyridine soluble material of a methanol:ether 1:1 extract of a freeze-dried preparation of ^{14}C glucose and ghosts. The radioactive counts per fraction are represented by the continuous line graph and carbohydrate per alternate fraction by the broken line graph.



Graph 31. Silicic acid/Hyflo column of the ether soluble material of a methanol:ether 1:1 extract of a freeze-dried preparation of ^{14}C glucose and ghosts inhibited with non-radioactive mercuric chloride. The radioactive counts and carbohydrate per fraction are represented by the continuous and broken line graphs respectively.



Graph 32. Silicic acid/Hyflo column of the pyridine soluble material of a methanol:ether 1:1 extract of a freeze-dried preparation of ^{12}C glucose and ghosts inhibited with radioactive mercuric chloride. The radioactive counts and carbohydrate per fraction are represented by the continuous and broken line graphs respectively.

individual solvents, viz., C:M:H_2O, 80:30:3 v/v, C:M, 1:2 v/v and dimethyl formamide.

The low quantity of radioactivity prevented radioactive scanning of the dried, chromatographed plates, but it was possible to spray them with sulphuric acid and char by heating. The developed plates are represented in Fig.28. Sphingomyelin and cerebroside standards were also applied to the plates on which X represents fractions 36-38.

In the C:M:H_2O solvent there were several spots in X, some of which were present in the sphingomyelin and cerebroside standards. Both standards gave more than one spot in this solvent system.

In the C-M 1:2 solvent, X chromatographed to give several lipid regions, some corresponding to those present in both the sphingomyelin and cerebroside standards. The most interesting spot was one which travelled about 0.5 cm. from the solvent front and which charred blue initially and finally black. This colour change was not observed in any of the standards but had previously been observed in other preparations of fractions 36-38 of silicic acid/Hyflo columns and also in the triphosphoinositide/glucose incubation preparation.

In the dimethyl formamide solvent system both cerebroside and X gave a large, poorly defined area at R_f value 0.85-0.9. Sphingomyelin gave a similarly poorly defined spot at R_f 0.5-0.6. This result had also been previously observed with other preparations.

The C-M 1:1 v/v extract of the methanol-dimethyl ether precipitate of experiment C had been retained since it was associated with considerable radioactivity it was therefore evaporated to dryness under reduced pressure and in a nitrogen atmosphere. The residue was successively extracted with acetone, diethyl ether and pyridine, and the radioactive

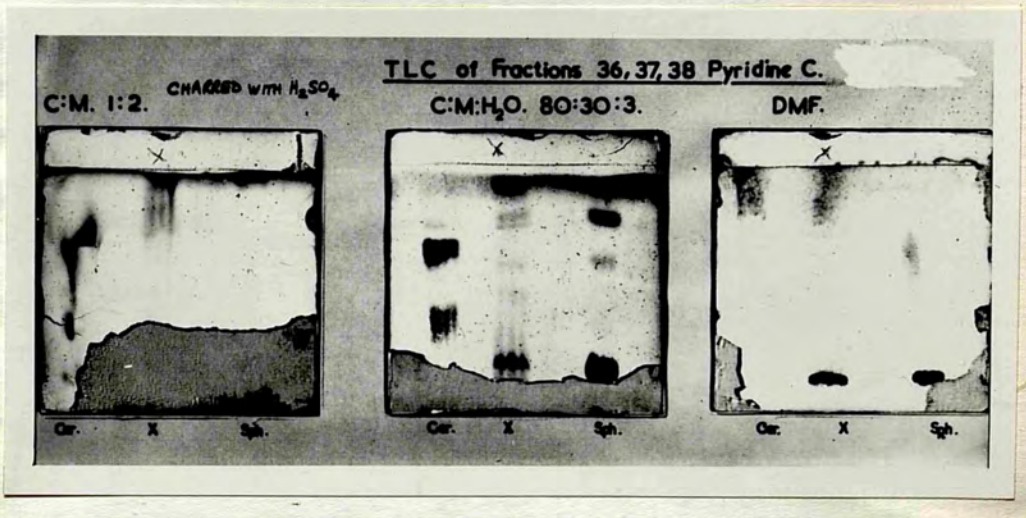


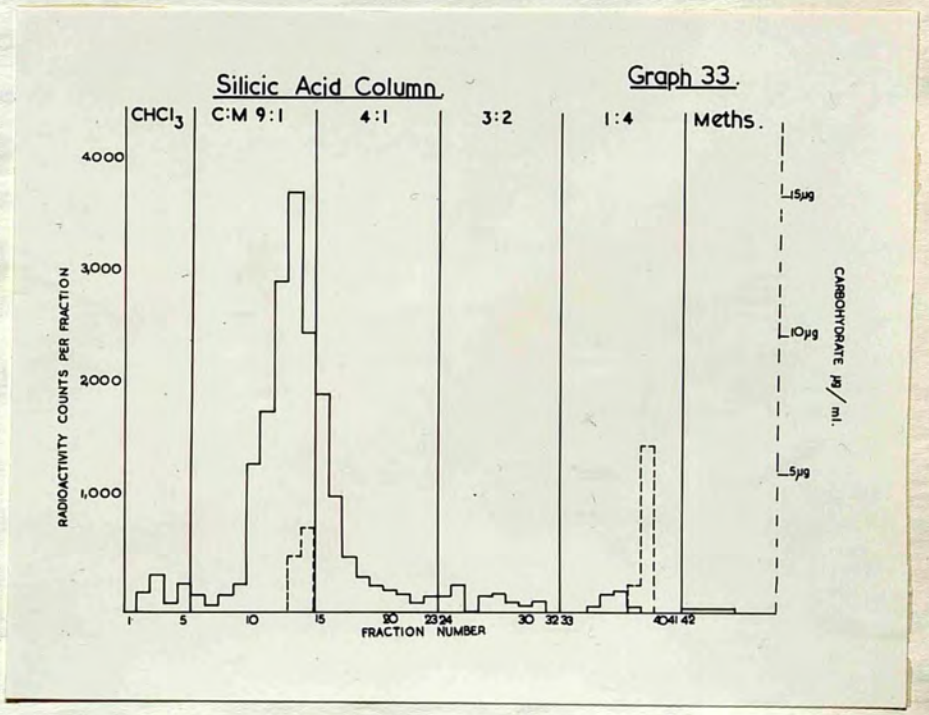
Fig.28. Thin layer chromatograms of Fractions 36, 37 and 38 of the silicic acid column of the pyridine extract of ghosts inhibited with radioactive mercury. The plates were chromatographed in C:M 1:2, C:M:H_2O, 80:30:3 and dimethyl formamide. The plates were developed by charring with 20% sulphuric acid. (X = Fractions 36, 37 and 38).

counts in each solvent were recorded.

Solvent:	M:C 1:1	Acetone	Ether	Pyridine
	94,800	28,880	9,000	26,000

The pyridine and acetone extracts were slightly coloured red-ish/brown with haem pigment derivatives. The pyridine extract was evaporated to dryness under reduced pressure and in a nitrogen atmosphere. The residue was dissolved in redistilled chloroform and applied to a silicic acid/Hyflo column. Radioactive counts and carbohydrate estimations were performed on all eluent fractions and the results are represented in Graph 33. A large peak of radioactivity was eluted in fractions 10-15, all of which were coloured with the 'haem' pigment. A very small quantity of radioactivity was eluted in fractions 36-38. Only two small regions of carbohydrate were present, neither of which was large enough to be of any real significance.

This result afforded a possible explanation for the very low quantity of radioactive mercury that had previously been extracted by the methanol-ether from the powdered ghosts. The ghosts and methanol-ether had remained in the refrigerator for a period of 3-4 days before separation. During this time the mercury may have become detached from any lipids it had been attached to and had been associated with the small quantity of haemoglobin present in the red cell ghosts. It was probable that the mercury had also had sufficient time to attack the protein material of the ghosts so that if these reactions involved greater affinity for mercury there could have been a loss of any 'mercury-lipid' material originally present. This



Graph 33. Silicic acid/Hyflo column of the pyridine soluble material of the chloroform:methanol 1:1 extract of a freeze-dried preparation of ^{12}C glucose and ghosts inhibited with radioactive mercuric chloride. The radioactive counts and carbohydrate per fraction are represented by the continuous and broken line graphs respectively.

possibility is made plausible from kinetic studies in which an initial large inhibition of glucose transfer by mercury passes off on further incubation. It was assumed the mercury, initially taken up by membrane elements was redistributed to haemoglobin and other intracellular molecules such as glutathione. Weed et al (1962) have estimated that the membranes take up less than 5% of mercury bound by the red cells as a whole.

The radioactive mercury although eluted in the same place as 'complexed' glucose was recovered in amounts several orders of magnitude lower than glucose. Since glucose complexing was near to 100% inhibited further experiments are needed to show whether mercury had reacted with the lipids during the incubation but had been lost during extraction (a possibility suggested above) or whether the mercury was blocking the formation of glucose complexes in some other way.

Reversible Inhibitors

Phloretin

The reversible inhibitor phloretin was used in an experiment where butanol and acid-butanol extractions were performed on both uninhibited and inhibited ghosts. 60.0 ml. of ghosts were used in each experiment and 10.0 ml. of a stock solution of ^{14}C glucose were added to each. The cells and glucose were incubated for one minute with shaking at ice-cold temperatures, and then extracted with ice-cold butanol. The butanol extracted the ghosts for a period of 18-24 hours in the refrigerator. The butanol was then filtered from the ghost residues and a second extraction with acid-butanol was performed on each residue. Radioactivity counts were taken of all four extracts with the following results :-

Experiment A - Uninhibited Cells

Stock ^{14}C Added	^{14}C Recovered in Butanol	^{14}C in Acid-Butanol
3,072,000 counts	941,000 counts	602,240 counts

Experiment B - Phloretin-Inhibited Cells

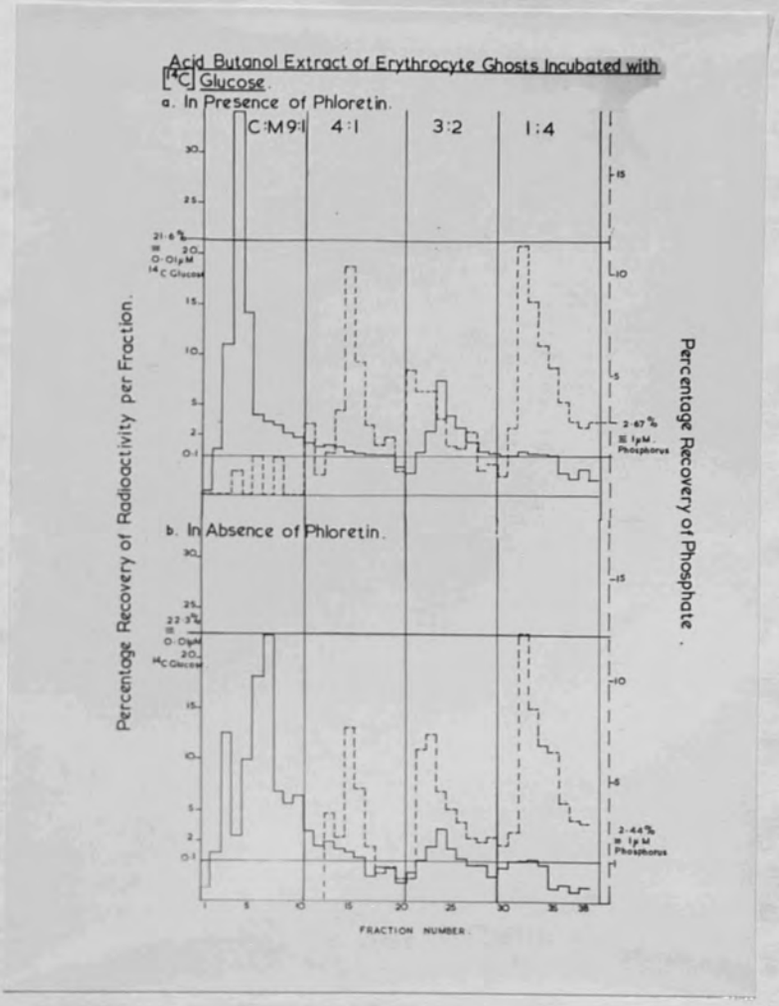
Stock ^{14}C Added	^{14}C Recovered in Butanol	^{14}C in Acid-Butanol
3,072,000 counts	461,090 counts	432,860 counts

All four extracts were evaporated to dryness under reduced pressure and in a nitrogen atmosphere for application to silicic acid/Hyflo columns. Radioactive counts and phosphate estimations were performed on all eluent fractions.

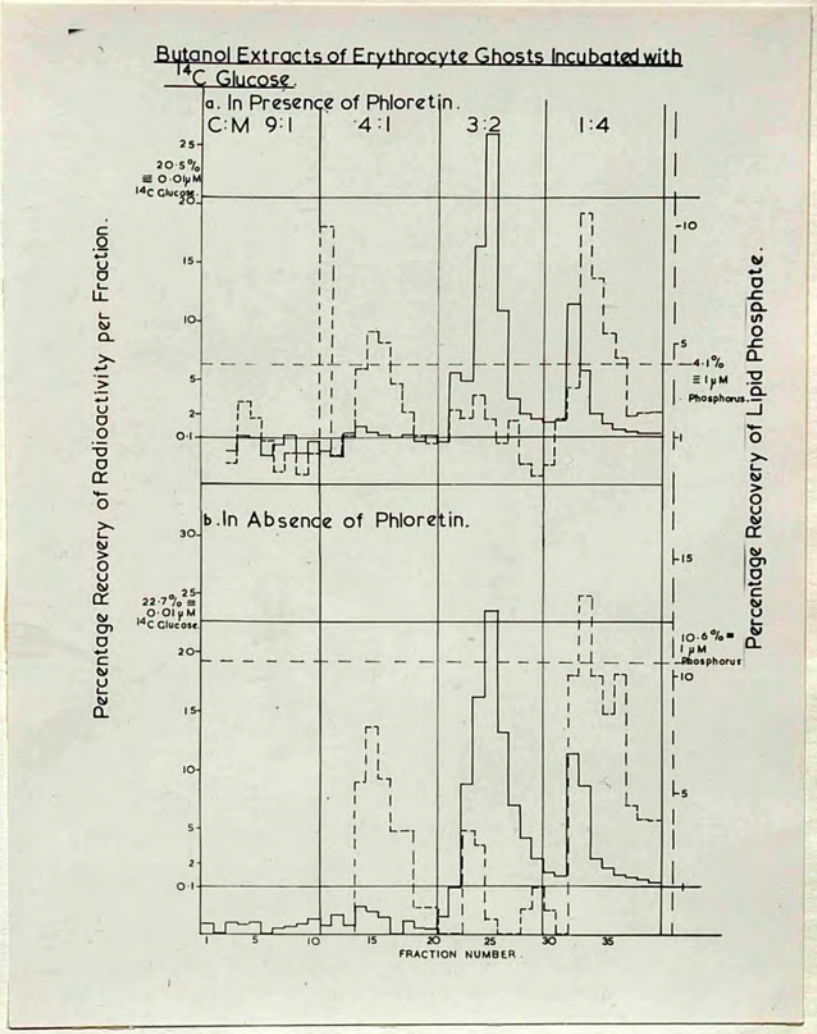
Graph 34 represents the results of the butanol extracts of Experiments A and B. In both extracts the radioactivity was eluted in two main regions : (a) Fractions 21-28 of the C-M 3:2 solvent, (b) Fractions 31-34 of the C-M 1:4 solvent. The phosphate elution patterns were also similar, with the exception of Fractions 1-10 where phosphate was only present in these fractions in experiment B, i.e., with phloretin. In both extracts the radioactivity peak in Fractions 31-34 was associated with a high phosphate content.

Graph 35 represents the results of the acid-butanol extracts. The radioactivity elution patterns were similar with a large peak at Fractions 2-7 and a smaller one at Fractions 21-27. The later peak present in the butanol extracts at Fractions 31-34 was absent from both acid-butanol extracts. The phosphate elution patterns were also similar with high recoveries in the C-M 4:1, 3:2 and 1:4 solvents in both cases. There was very little in Fraction 1-10 in both extracts. Fractions 5 and 6 of both were strongly contaminated with haemoglobin.

Eluent fractions from all four columns were selected for thin-layer chromatography on the larger silica gel G plates



Graph 35. Silicic acid/Hyflo columns of acid:butanol extracts of aqueous preparations of :-
A. Ghosts and ^{14}C glucose and phloretin.
B. Ghosts and ^{14}C glucose.
The percentage recoveries of radioactivity and of phosphate per fraction are represented by the continuous and broken line graphs respectively.



Graph 34. Silicic acid/Hyflo columns of butanol extracts of aqueous preparations of :-

A. Ghosts and ^{14}C glucose and phloretin.

B. Ghosts and ^{14}C glucose.

The percentage recoveries of radioactivity and of phosphate per fraction are represented by the continuous and broken line graphs respectively.

and run in C-M-H_2O 80:30:3. The plates were subsequently scanned for radioactivity with the following results :-

Experiment	Fractions	Rf values of Radioactivity peaks	Rf value of ^{14}C Glucose
A. Butanol	32	0.09, 0.24	0.09 - 0.11
B. Butanol	3,4,5 (Pooled)	0.65	"
	13,14,15(")	0.15,0.31,0.65	"
	22,23(")	0.09	"
	27,28(")	0.11	"
A. Acid-Butanol	3	0.95	"
	9	0.58	"
B. Acid-Butanol	3	0.1	"
	9	0.6	"
	13	0.52	"

The plates were developed in iodine and ninhydrin, but the contamination by haem pigment and high degree of heterogenity prevented any analysis of the results. Since the acid-butanol extracts were later found to contain some artifact., the results of thin-layer plates had to be largely discounted.

However, the radioactivity elution patterns from the silicic acid columns afforded three possible explanations for the action of phloretin on the red cell ghosts. The total glucose in Fractions 21-27 and Fractions 31-34 of the butanol extracts appeared to be identical in both experiments, although the phloretin may have delayed the elution of phosphate in the 31-34 region by one fraction. The three possibilities were, therefore :-

(i) Under the described experimental conditions inhibition had failed to take place.

(ii) The component of the membrane involved in glucose transport had not been extracted by the butanol solvent.

(iii) Phloretin inhibited the red cell sugar transport system without actually preventing the formation of complex material.

If the latter were the case, then the action of phloretin would not be in accordance with the generally accepted views that reversible inhibitors combine directly with the substrate site.

Stilboestrol

Stilboestrol reversibly inhibits sugar transport in the erythrocyte. A ^{14}C stilboestrol preparation was obtained from the Radiochemical Centre, Amersham. The stilboestrol was a 2 μ molar solution in benzene, since it deteriorates in any other solvent. For the experiment, the benzene was evaporated under reduced pressure and in a nitrogen atmosphere, and the stilboestrol was taken up in absolute alcohol and diluted with saline. Two portions of red cell ghosts were used, to each of which were added 10.0 ml. of stock stilboestrol solution. No sugar was added since only the behaviour of stilboestrol with respect to the membrane lipids was to be observed. 50.0 ml. of ghosts were used in each experiment and the concentration of stilboestrol was 0.25×10^{-6} moles. The ghosts and stilboestrol were incubated at ice-cold temperatures for one minute with shaking, then each was extracted with different lipid solvents also at ice-cold temperatures.

Experiment A

The ghosts were initially extracted with ethyl acetate for a period of one hour. After filtration the residue was then extracted with chloroform which, on filtration, yielded two phases, a lower one of chloroform and an upper of water. The residue was finally extracted with a C-M-Acid solvent. Radioactive counts were taken of each extract and the quantities in each were :-

^{14}C Stilboestrol added		
2,415,000	^{14}C ethyl acetate	545,648
	^{14}C chloroform phase	901,020
	^{14}C water phase	5,928
	^{14}C C-M-Acid	95,280

Experiment B

The ghosts were extracted with ice-cold butanol only, for a period of one hour.

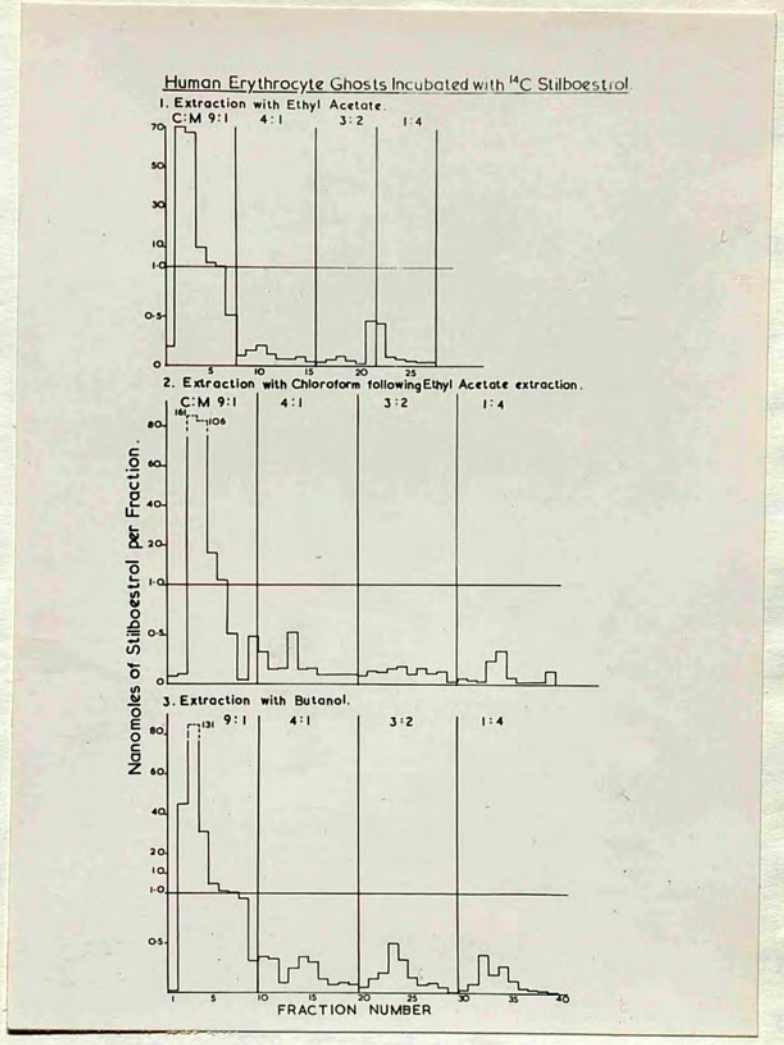
^{14}C stilboestrol added	^{14}C Butanol
2,415,000	810,700

The ethyl acetate and chloroform extracts of Experiment A and the butanol extract of Experiment B were evaporated to dryness under reduced pressure and in a nitrogen atmosphere, and the residues dissolved in redistilled chloroform for application to silicic acid/Hyflo columns. Radioactive counts of each fraction were recorded and the results are represented on Graph 36.

There was a large region of radioactivity in Fractions 1-10 in all three extracts which corresponded to the elution of free stilboestrol. There were some small, other regions of radioactivity, particularly in the butanol extract, but all were well below 1 μ mole of stilboestrol.

Fractions from all three columns were selected for thin-layer chromatography on the larger silica gel G plates and in a C-M-H_2O 80:30:3 (v/v) tank. The results of subsequent radioactive scanning of the dry plates were as follows :-

Extract	Fractions	Rf value ^{14}C	Interpretation
Free ^{14}Stilboestrol		0.75 - 1.05 Peak 0.85	
Ethyl Acetate	2	0.65 - 0.95 Peak 0.8	free stilboestrol
"	6	0.75 - 1.05 Peak 0.83	free stilboestrol
"	9,10,11 (pooled)	0.75 - 0.89 Peak 0.8	free stilboestrol
	"	Shoulder 0.65	non-free "



Graph 36. Silicic acid/Hyflo columns of -
1. Ethyl Acetate, 2. Chloroform and 3. Butanol extracts of an aqueous preparation of ghosts incubated with ^{14}C stilboestrol. The radioactive stilboestrol per fraction is represented.

Extract	Fractions	Rf value ^{14}C	Interpretation
Ethyl Acetate	21,22,23 (pooled)	0.7 - 0.9	
		Peak 0.77	Free stilboestrol
Chloroform	9,10	0.7 - 0.9	
		Peak 0.83	free stilboestrol
"	13,14,15 (pooled)	0.55- 0.9	
		Peak 0.7	non-free "
Butanol	7, 8 (pooled)	0.55 - 0.95	
		peaks (i)0.83	free "
		(ii)0.6	non-free "
"	9,10,11 (pooled)	0.48 - 0.95	
		Peak 0.76	free stilboestrol
"	13,14,15 (pooled)	0.42 - 0.93	
		peaks (i)0.65	non-free "
		(ii)0.45	" " "
"	22,23,24 (pooled)	0.25 - 0.8	
		plateau 0.4-0.75	" "
"	32,33,34 (pooled)	0.35 - 0.8	
		plateau 0.38-0.64	" " "

Figs. 29a, 29b and 29c show all three plates after development in an iodine vapour tank. All samples were heterogeneous and analysis was consequently not possible. An experiment was performed to see if stilboestrol taken into the cell was displaced by glucose as might be expected from a competitive inhibitor.

The principle of the experiment was based on the incubation of blood with glucose and radioactive stilboestrol, then to centrifuge the cells and to measure the radioactivity in the supernatant and cells. A series of glucose concentrations was used to observe the effect on the subsequent radioactivity distribution. Two series of experiments were set up where two different concentrations of stilboestrol were used : (a) 0.1 ml. stock ^{14}C stilboestrol, (b) 0.2 ml. stock ^{14}C stilboestrol.

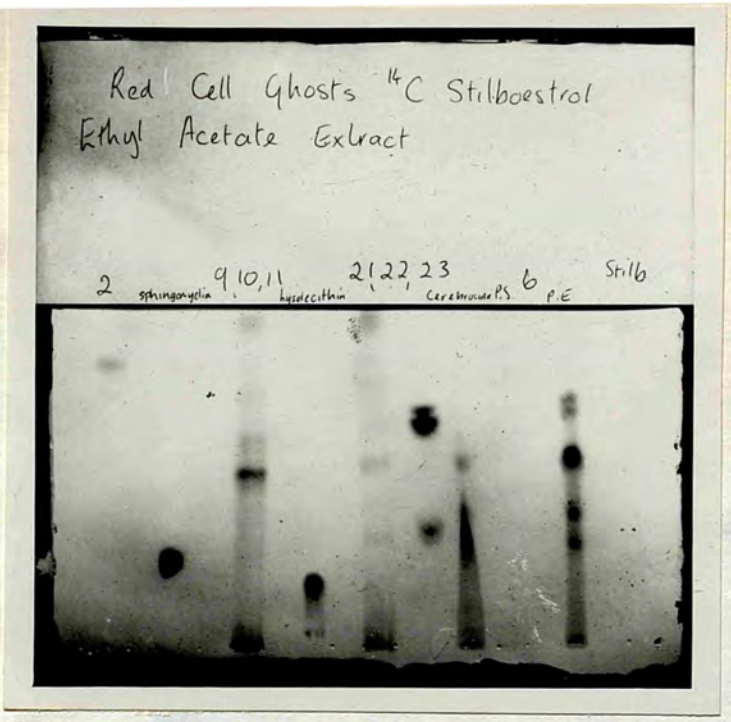


Fig.29a. Thin layer chromatogram of Fraction 2, Fractions 9-11 and Fractions 21-23 of the silicic acid column of the ethyl acetate extract of the stilboestrol inhibited ghosts. The plates were chromatographed in C:M:H_2O, 80:30:3 and developed in a saturated iodine tank.

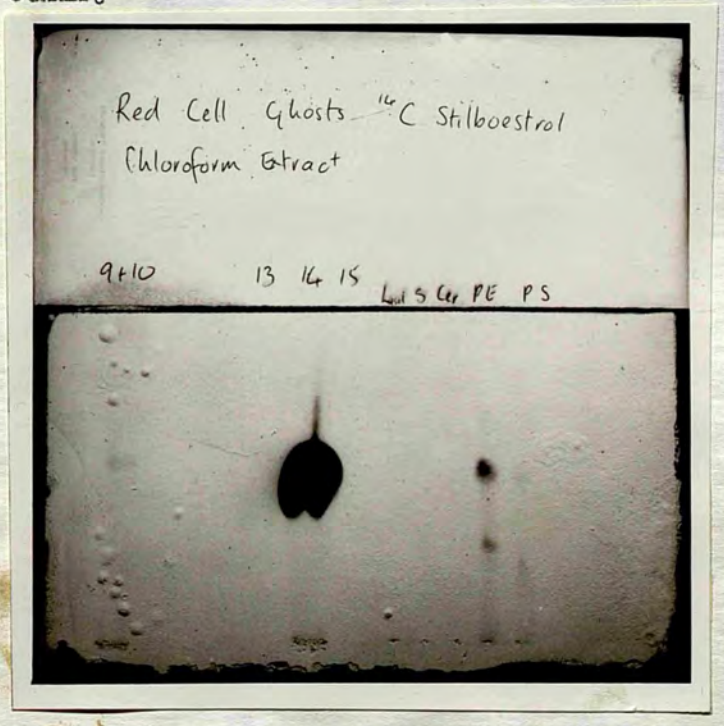


Fig.29b. Thin layer chromatogram of Fractions 9 and 10 and Fractions 13-15 of the silicic acid column of the chloroform extract of the stilboestrol inhibited ghosts. The plates were chromatographed in C:M:H_2O, 80:30:3 and developed in a saturated iodine tank.

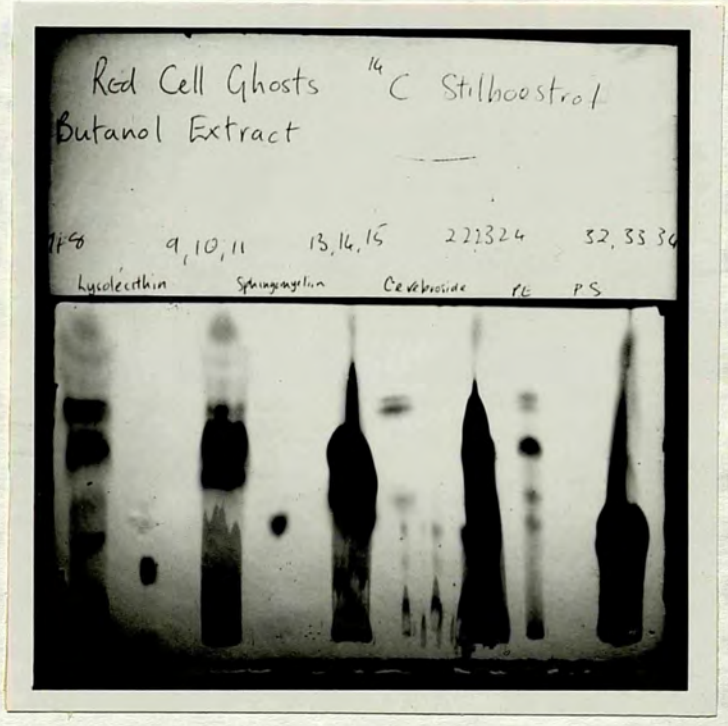


Fig.29c. Thin layer chromatogram of Fractions 7 & 8, Fractions 9-11, Fractions 13-15 and Fractions 22-24 of the silicic acid column of the butanol extract of the stilboestrol inhibited ghosts. The plates were chromatographed in C:M:H_2O, 80:30:3 and developed in a saturated iodine tank.

All samples were incubated for 10 minutes, after which time they were centrifuged for 5 minutes at 3,000 r.p.m. and the resulting supernatant removed. Radioactive counts of cells and supernatant were recorded and the results were as follows :-

Experiment A - 0.1 ml. stilboestrol

ml. Glucose	ml. cells	ml. buffer	Cells cts/ml.	Supt.cts/ml.	Ratio
4.0	0.3	0.1	223,000	672	330
2.5	0.3	1.6	214,000	1,094	195
1.5	0.3	2.6	257,000	1,320	192
0.5	0.3	3.6	327,000	1,094	193
-	0.3	4.1	237,000	1,944	122

Experiment B - 0.2 ml. stilboestrol

ml. Glucose	ml. cells	ml. buffer	Cells cts/ml.	Supt.cts/ml.	Ratio
4.0	0.3	0.1	409,000	950	430
2.5	0.3	1.6	333,000	1,122	296
1.5	0.3	2.6	457,000	1,510	300
0.5	0.3	3.6	463,000	1,902	240
-	0.3	4.1	448,000	2,236	200

The concentration of the glucose solution was 15.2mg/10ml. in both experiments.

From the results it appeared that the lower the concentration of glucose the more stilboestrol was present in the supernatant, which was in effect the reversal of what would be expected. A possible explanation was that the stilboestrol associates more strongly with cell lipids (or proteins) in the presence of glucose so that the higher the concentration of glucose in the cells, the larger amount of stilboestrol taken up and the less in the supernatant.

It was also possible that no inhibition had occurred under these experimental conditions, in any event the high

cell/medium ratio meant that this inhibitor was not valuable in specifically labelling sites of glucose uptake in the cell membrane.

d. Experiments with Sheep Erythrocyte Ghosts

The sheep erythrocyte ghosts were prepared by the same method as the human erythrocyte ghosts.

25.0 ml. of sheep ghosts were incubated with 15.0 ml. of stock ^{14}C glucose solution and 5.0 ml. of saline for one minute at ice-cold temperatures. They were then extracted for one hour with ice-cold butanol.

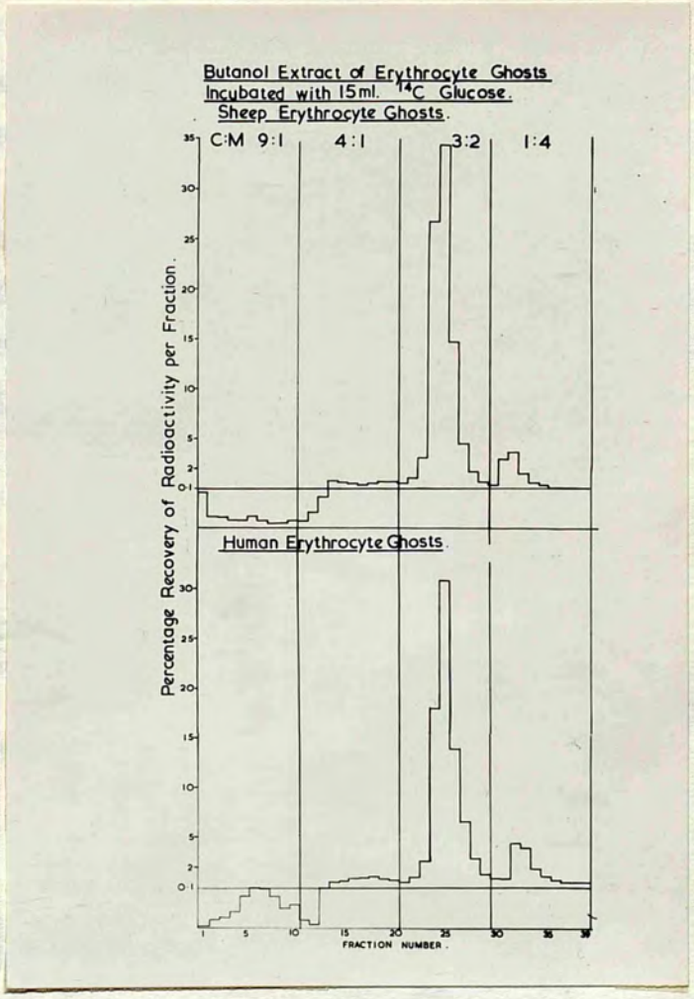
In conjunction with this experiment a similar extraction was performed on an incubation media of 30.0 ml. of human ghosts, 15.0 ml. of stock ^{14}C glucose solution and 15.0 ml. of saline.

After the period of extraction the butanol was filtered from each experiment and the radioactivity in the extracts recorded.

	^{14}C stock glucose added	^{14}c in Butanol
Sheep Ghosts	1,850,000 counts	435,200 counts
Human Ghosts	1,850,000 "	575,360 "

The butanol extracts were evaporated to dryness under reduced pressure and in a nitrogen atmosphere. The residues were dissolved in redistilled chloroform for application to silicic acid/hyflo columns. The percentage radioactivity in each eluent fraction was recorded and the results are represented in Graph 37.

The distributions of radioactivity in the eluent fractions of both extracts were very similar with only one region of radioactivity present in fractions 22-28. All fractions had radioactivity above the background count. The region of radioactivity in fractions 22-28 corresponded to the elution of free glucose and radioactive scanning of thin layer plates of these fractions gave only one region of radioactivity at the origin. Dimethylformamide was not



Graph 37. Silicic acid/Hyflo columns of butanol extracts of aqueous preparations of sheep ghosts and ^{14}C glucose and of human ghosts and ^{14}C glucose. The percentage recovery of radioactivity per fraction is represented.

being used for thin layer chromatography at this earlier stage and the plates were developed in a C:M:H_2O, 80:30:3 v/v solvent system.

In a later experiment sheep ghosts were incubated with glucose and freeze dried before extraction with a series of lipid solvents. The effect of increased glucose concentration in the incubation media was observed and a comparison made of these results with those from human ghosts under similar experimental conditions. The freeze dry phials were set up for both sheep and human ghosts as follows :-

Experiment A

Three phials each containing :-

1.7 ml. ghosts
0.1 ml. ^{12}C glucose solution (14.4 mgm. in 5.0 ml. saline)
0.2 ml. stock ^{14}C glucose

Experiment B

Three phials each containing :-

1.7 ml. ghosts
0.1 ml. ^{12}C glucose solution (50.4 mgm. in 5.0 ml. saline)

Experiment C

Three phials each containing :-

1.7 ml. ghosts
0.1 ml. ^{12}C glucose solution (176.4 mgm. in 5.0 ml. saline)

The final concentrations of glucose in Experiments A, B and C were 1, 3 and 10mMolar respectively.

All solutions were at ice-cold temperatures and the incubation period was for one minute with thorough mixing of the solutions. When the ghosts were completely dry they were extracted with methanol-ether 1:1 v/v and

radioactive counts of the extracts were recorded. The methanol-ether was evaporated to dryness under reduced pressure and in a nitrogen atmosphere and the residues were successively extracted with acetone, diethyl ether and pyridine. Radioactive counts of each extract were recorded.

Sheep Ghosts

Experiment A	M-E	1,118,005	counts
	Acetone	338,880	"
	Ether	333,795	"
	Pyridine	333,000	"
Experiment B	M-E	724,850	"
	Acetone	189,000	"
	Ether	112,980	"
	Pyridine	303,840	"
Experiment C	M-E	666,346	"
	Acetone	150,720	"
	Ether	22,000	"
	Pyridine	491,310	"

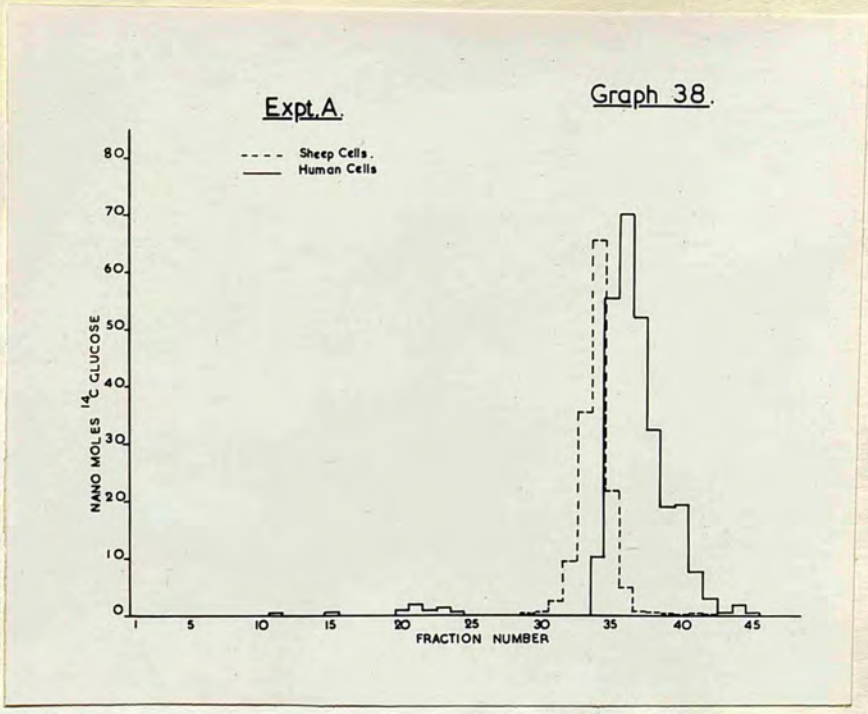
Human Ghosts

Experiment A	M-E	464,040	counts
	Acetone	60,750	"
	Ether	57,420	"
	Pyridine	348,000	"
Experiment B	M-E	509,480	"
	Acetone	36,750	"
	Ether	90,500	"
	Pyridine	285,000	"
Experiment C	M-E	484,100	"
	Acetone	29,760	"
	Ether	70,250	"
	Pyridine	270,000	"

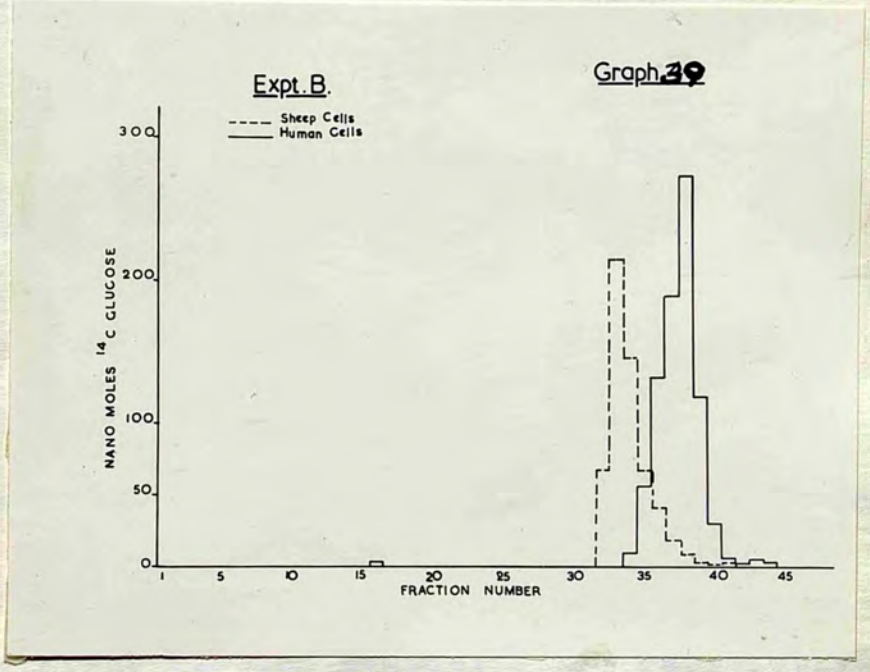
The pyridine extracts of all six experiments were evaporated to dryness under reduced pressure in a nitrogen atmosphere and the residues were dissolved in redistilled chloroform. There was no insoluble material in any of the extracts. The chloroform extracts were applied to silicic acid/hyflo columns and radioactive counts of all eluent

fractions recorded. The results are represented in Graphs 38, 39 and 40. In the sheep ghosts the radioactivity was all in fractions 30-36 with the peak at fraction 33. In the human ghosts the radioactivity was in fractions 34-41 with the peak at fractions 37-38. Material from all six peaks was evaporated to dryness and applied to thin layer plates in redistilled chloroform. All plates were developed in a dimethylformamide solvent and when dry were scanned for radioactivity. The peak material from the sheep cell extracts gave only one region of radioactivity which was at the origin. The peak material from the human red cell extracts gave two regions of radioactivity one of which was at the origin and the other at Rf. 0.85. In both cells increased glucose concentration produced a corresponding increase in the quantity of sugar associated with the radioactive peaks.

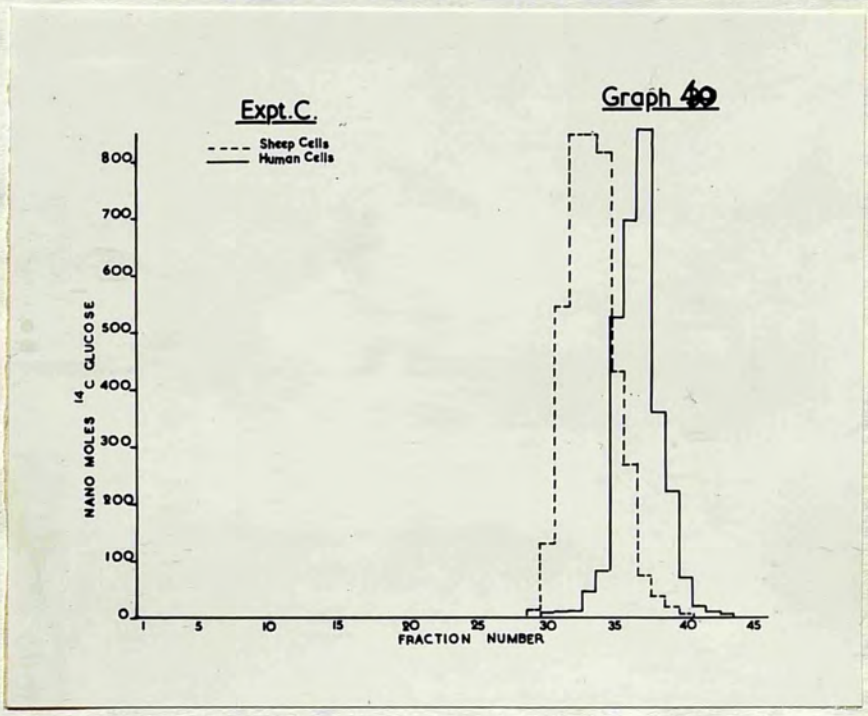
The sheep cell peak material did not therefore consist of any of the complex material Rf. 0.85 and appeared to consist of free glucose only. This result does not confirm the involvement of this peak material in the sugar transfer of the human red cell but indicates its absence from sheep red cells which do not possess a sugar transfer system. On the other hand it is difficult to understand why radioactivity is found in the pyridine extract from sheep ghosts to nearly the same amount as from human ghosts, unless it is being solubilised by some lipid component.



Graph 38. Silicic acid/Hyflo columns of the pyridine soluble material of methanol:ether 1:1 extracts of sheep and human ghosts incubated in a 1 m Molar glucose medium. The radioactive sugar per fraction is represented.



Graph 39. Silicic acid/Hyflo columns of the pyridine soluble material of methanol:ether 1:1 extracts of sheep and human ghosts incubated in a 3 m Molar glucose medium. The radioactive sugar per fraction is represented.



Graph 40. Silicic acid/Hyflo columns of the pyridine soluble material of methanol:ether 1:1 extracts of sheep and human ghosts incubated in a 10 m Molar glucose medium. The radioactive sugar per fraction is represented.

e. The Effects of Inhibitors on Artificial Systems
Phenolphthalein

Phenolphthalein reversibly inhibits the red cell sugar transfer system. (Forsling and Widdas 1966). A study was made of the effect of phenolphthalein on the artificial triphosphoinositide-glucose preparation.

The effect of phenolphthalein on the lipid standards was first recorded by incubating 1.0mgm of lipid standard with 1.0mgm of phenolphthalein in a pyridine medium for one hour at 22°C. The lipids were then applied to the larger thin layer silica gel plates and developed in a C:M:H_2O 80:30:3 solvent. The pure lipids were also applied to the plate. The plate was first developed in a saturated iodine tank to detect the unsaturated lipids. When most of the iodine had faded the plate was sprayed with an alcoholic sodium hydroxide solution to detect the phenolphthalein. Fig. 30 shows the results. The phenolphthalein did not run with any of the lipid standards but in each sample ran to an R.f. value of 0.8. The phenolphthalein, therefore, did not appear to associate with any of the lipid standards used.

The effect of phenolphthalein on the triphosphoinositide and in particular the triphosphoinositide-glucose preparation was recorded by incubating 1.0mgm of triphosphoinositide with 1.0mgm of a stock ^{14}C glucose solution in pyridine and adding 1.0mgm of phenolphthalein to the incubating medium. The incubation period was for one hour at 22°C. Several samples were applied to the plate :-

Triphosphoinositide + phenolphthalein
Triphosphoinositide alone
^{14}C glucose alone
^{14}C glucose + phenolphthalein
A = ^{14}C glucose + triphosphoinositide
B = ^{14}C glucose + triphophoinositide + phenolphthalein

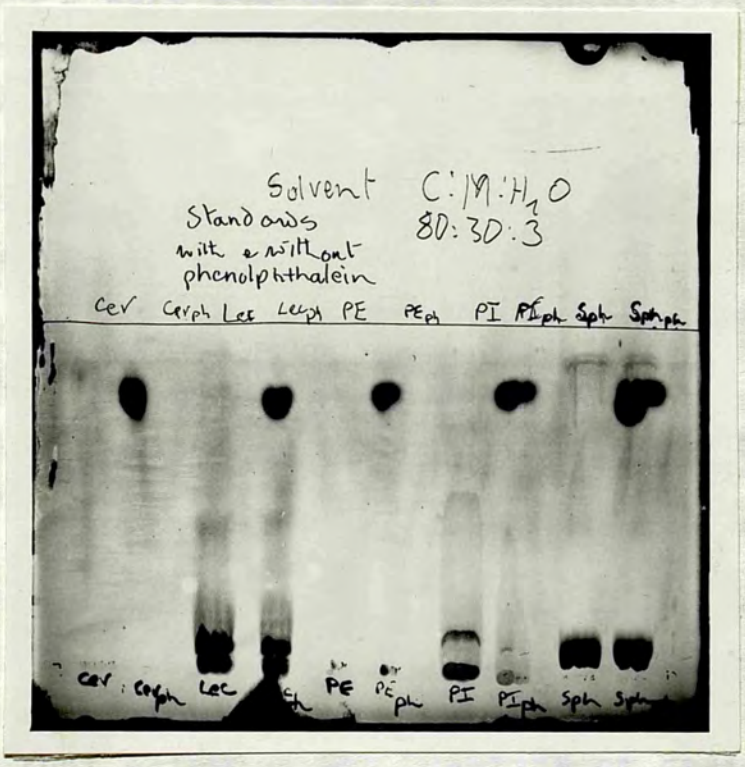


Fig.30. Thin layer chromatogram of lipid standards with and without phenolphthalein. The plates were chromatographed in C:M:H_2O, 80:30:3 and developed in a saturated iodine tank followed by spraying with alcoholic sodium hydroxide.

The plates were run in two solvent systems -
(a) C:M:H_2O 80:30:3, and
(b) Dimethylformamide.

The plates were developed first in a saturated iodine tank and were later sprayed with alcoholic sodium hydroxide solution. A and B were scanned for radioactivity. Figs. 31(a), (b) and (c) show the results in the C:M:H_2O solvent system. The phenolphthalein did not run with any of the lipid spots but travelled to the R_f value previously observed. The radioactivity scanning patterns were similar in A and B and corresponded to that of free glucose.

Figs. 32, 33 and 34 show the results in the dimethylformamide solvent. The plate did not develop in the iodine vapour tank but the phenolphthalein spots did develop after spraying with the alcoholic sodium hydroxide solution. In both the triphosphoinositide samples, where phenolphthalein was present, the latter was held back from the solvent front to an R_f value of 0.8 - 0.85. The phenolphthalein incubated with glucose alone travelled to the solvent front. Radioactive scanning showed that although the quantity of radioactivity applied in sample B was less than in sample A, the relative quantity of material at R_f value 0.8 - 0.85 was not reduced in the presence of phenolphthalein although the phenolphthalein did travel with this material. The phenolphthalein did not therefore appear to inhibit the formation of the complex material but travelled with it. The phenolphthalein may have been associating with the glucose by e.g., hydrogen bonding but not preventing it from complexing with the triphosphoinositide.

Triton X-100

The detergent Triton X-100 irreversibly inhibits glucose transport across the red cell membrane. (Hunter 1964).

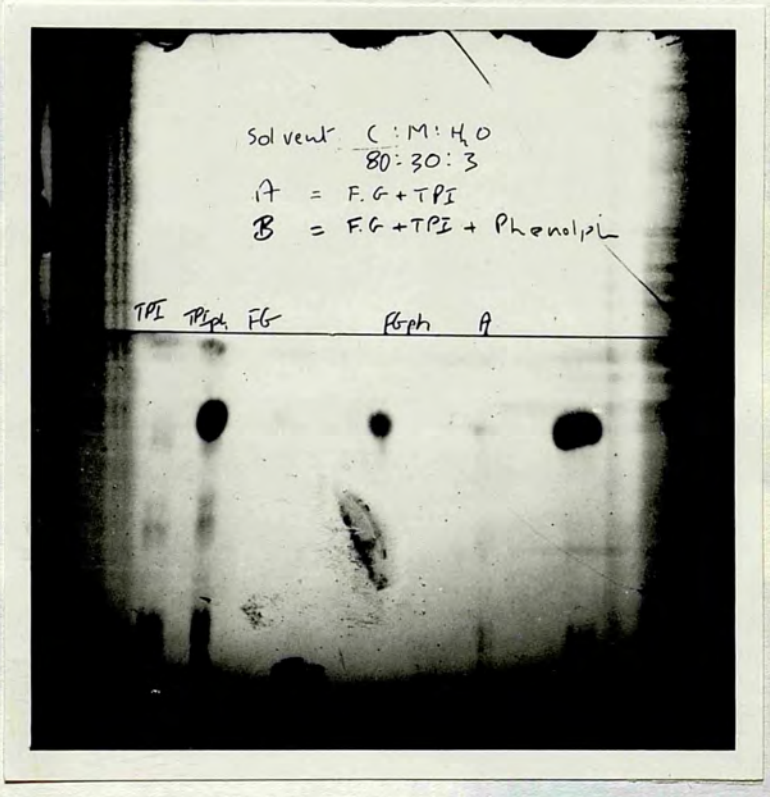


Fig.31a. Thin layer chromatogram showing the effect of phenolphthalein on the triphosphoinositide : + ^{14}C glucose preparation. The plate was chromatographed in C:M:H_2O, 80:30:3 and developed in a saturated iodine tank followed by spraying with alcoholic sodium hydroxide.

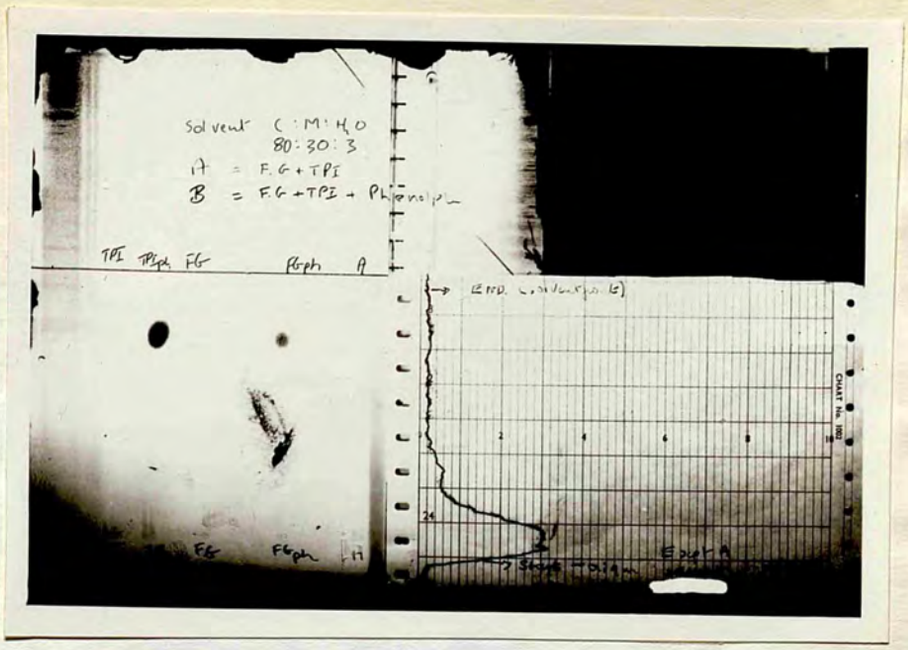


Fig.31b. Radioactive scan of preparation A- ^{14}C glucose + triphophoinositide. The plate was chromatographed in C:M:H_2O 80:30:3 and developed in a saturated iodine tank followed by spraying with alcoholic sodium hydroxide.

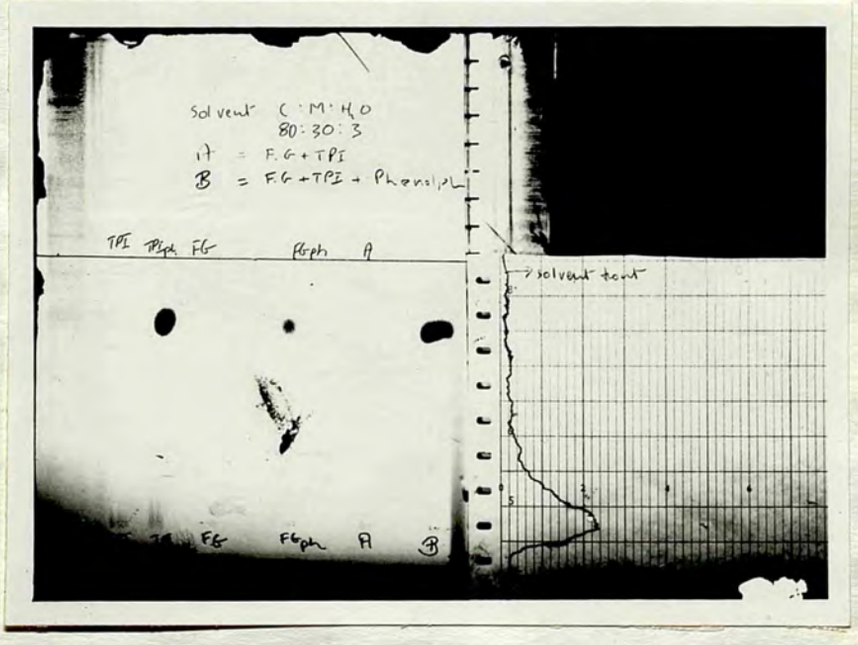


Fig.31c. Radioactive scan of preparation B- ^{14}C glucose + triphosphoinositide + phenolphthalein. The plate was chromatographed in C:M:H_2O, 80:30:3 and developed in a saturated iodine tank followed by spraying with alcoholic sodium hydroxide.

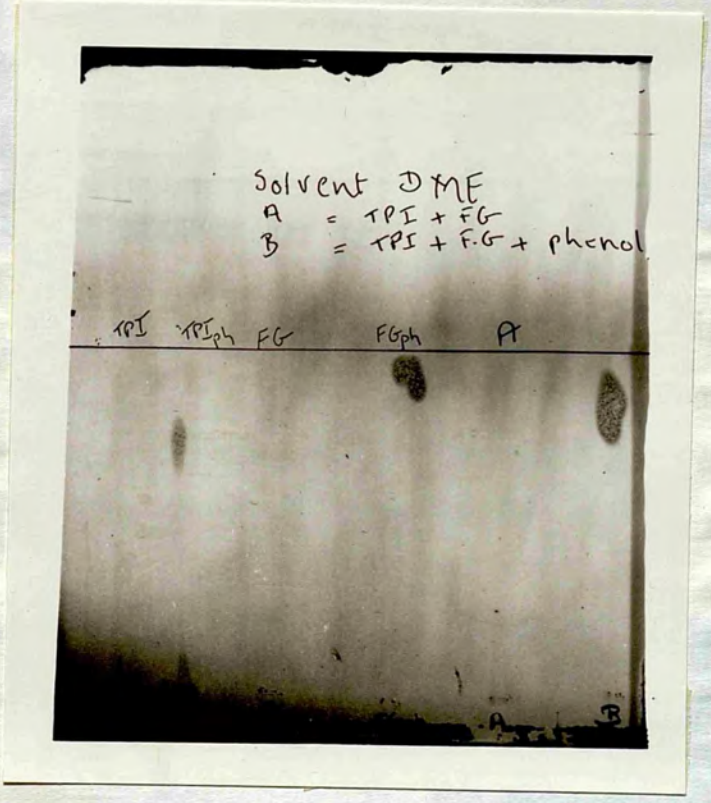


Fig.32. As Fig.31 but chromatographed in dimethyl formamide and developed in a saturated iodine tank followed by spraying with alcoholic sodium hydroxide.

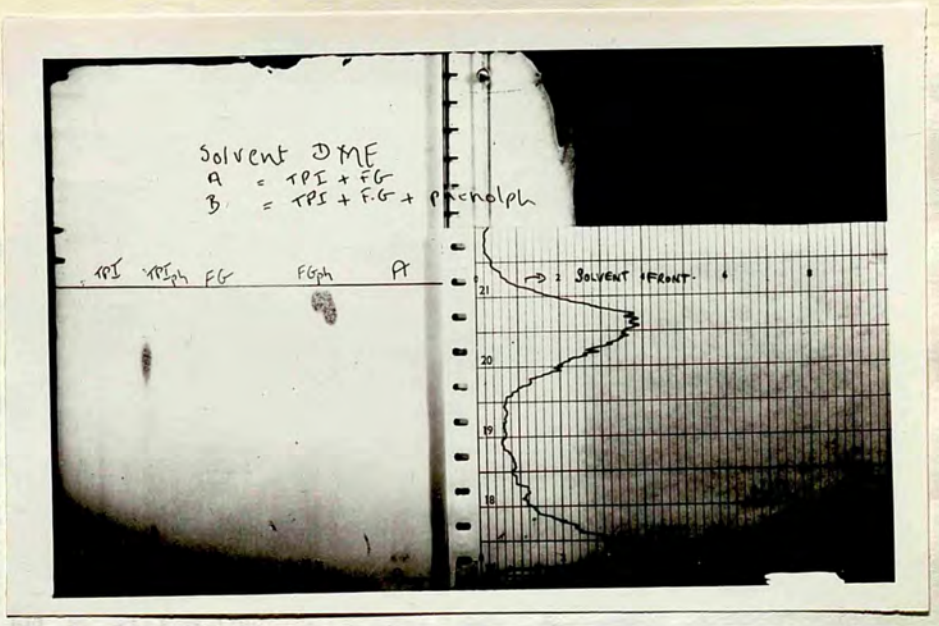


Fig.33. Radioactive scan of preparation A-^{14}C glucose + triphosphoinositide. The plate was chromatographed in dimethyl formamide and developed by spraying with alcoholic sodium hydroxide.

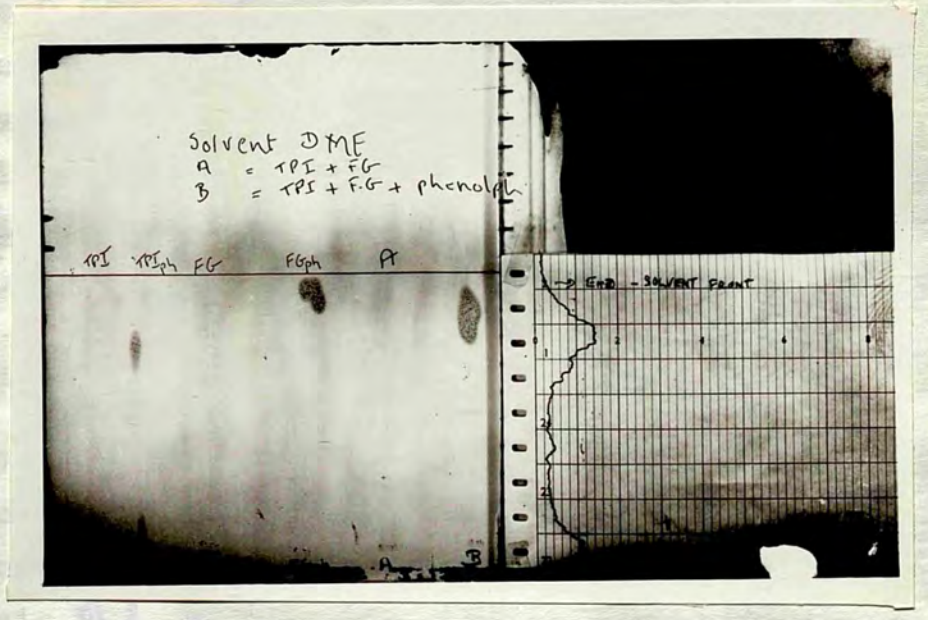


Fig.34. Radioactive scan of preparation B-^{14}C glucose + triphosphoinositide + phenolphthalein. The plate was chromatographed in dimethyl formamide and developed by spraying with alcoholic sodium hydroxide.

0.1 m/l of Triton X-100 was taken up in 2.0 m/l of pyridine. To this solution 2.0 mgm of triphosphoinositide and 0.1 m/l of a stock ^{14}C glucose in pyridine were added. The materials were incubated in the pyridine media for one hour at 22°C. Three standard solutions of (a) ^{14}c Glucose plus triphosphoinositide in pyridine, (b) ^{14}C glucose plus Triton X-100 in pyridine, and (c) ^{14}C glucose alone in pyridine were prepared. After the incubation period the pyridine was evaporated under reduced pressure in a nitrogen atmosphere and all four residues were applied in redistilled chloroform to a 20x20cm. silica gel G thin layer plate. The plate was chromatographed in the dimethylformamide solvent. When dry, the samples were scanned for radioactivity. The results are represented in Fig. 35. The free glucose and the glucose plus Triton X-100 samples gave only one region of radioactivity which was at the origin. (Rf value 0.05). The triphosphoinositide plus glucose sample gave two regions of radioactivity one at the origin Rf. 0.05 and a larger second one at Rf. 0.8. In the triphosphoinositide plus Triton X-100 sample this larger peak at Rf. 0.8 was not present but there was an irregular distribution of radioactivity extending from the origin to Rf. value 0.7. The largest peak of radioactivity was at the origin.

It appeared that the Triton X-100 had prevented the formation of the complex material of Rf value 0.85 but the lipid material alone was visible under ultra-violet light at this Rf. value. A similar ultra-violet visible spot was seen in the other triphosphoinositide sample.

Mercuric Ions

Mercuric ions irreversibly inhibit the sugar transfer system in the red cell although the degree of inhibition falls off rapidly after 30 mins. as the mercuric ions enter the

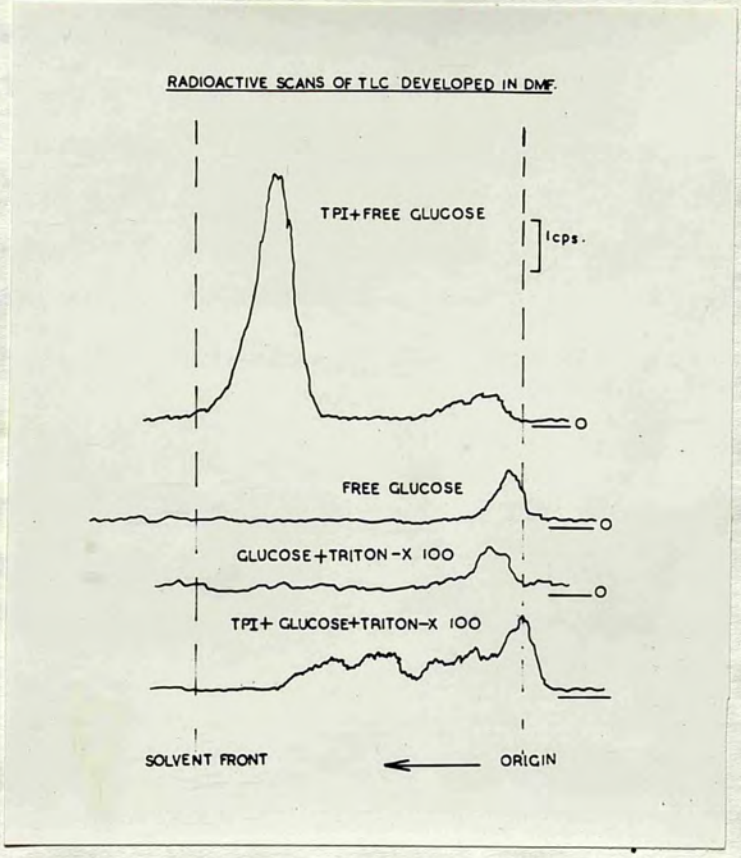


Fig.35. Radioactive scans showing the effect of Triton X-100 on the ^{14}C glucose + triphosphoinositide preparation. The plate was chromatographed in dimethyl formamide.

red cell haemoglobin. Since the mercuric ions were insoluble in the pyridine solvent an attempt was made to introduce them into the triphosphoinositide material in an ether-water partition phase system. 1.0 mgm of mercuric chloride was dissolved in 2.0 m/l of distilled water and this solution was shaken up with a diethyl-ether solution of triphosphoinositide (1.0 mgm in 2.0 m/l). The ether layer was removed and evaporated to dryness. The residue was dissolved in 0.1 m/l of pyridine and 0.1 m/l of a stock ^{14}C glucose solution in pyridine added to it. The resulting pyridine solution was incubated for one hour at 22°C. The pyridine was then evaporated to dryness under nitrogen and the residue applied in redistilled chloroform to a 20x20 cm thin layer silica gel G plate. An uninhibited triphosphoinositide plus ^{14}C glucose sample was also applied to the plate. The plate was chromatographed in a dimethylformamide solvent and, when dry, it was scanned for radioactivity. The radioactivity pattern was unchanged in the sample prepared with mercuric chloride. The two radioactivity peaks at R_f values 0.05 and 0.85 were unaltered. It was later found that the mercuric chloride had probably all remained in the water phase.

Discussion

Lipid solvent extractions on aqueous preparations.

For the proposed experiments a solvent system with the following properties was desirable :-

(a) High extraction yield of membrane lipids.
(b) Negative or low extraction of haem pigment.
(c) Solubilisation of glucose-lipid complexes without breakdown.

The conventional lipid extraction solvent of C-M 2:I (Folch, I957) complied with the first three requirements but was not a suitable solvent for glucose. Butanol was a better solvent for glucose, but its high boiling point involved long, undesirable periods of evaporation. The acidified solvents extracted the more polar lipids and some of the proteo-lipids, but they were strongly contaminated with haem pigment. The acidic conditions also promoted the production of artifact material resulting from a reaction between glucose and the alcohol. It was not possible to resolve the radioactivity associated with the artifact material into two fracions of mobilities, but it was unlikely that all the radioactivity was attributable to artifact.

The isopropanol-chloroform II:7 v/v (Rose and Oklander) solvent system proved to be a more suitable extraction solvent for the experimental conditions. There was a high recovery of radioactivity and a high lipid yield in the extracts, which were usually uncontaminated with haem pigment. The periods of lipid solvent evaporation were short, decreasing the possibilities of breakdown of any complex material present.

These factors were reflected in the radioactivity elution patterns of the silicic acid/Hyflo columns of the chloroform-methanol, butanol and isopropanol-chloroform extracts. The chloroform-methanol extracts gave little indication of the extraction of any radioactivity other than free glucose, which was eluted in the chloroform-methanol 3:2 v/v solvent. In butanol and isopropanol extracts radioactivity was eluted in the chloroform-methanol 4:1, 3:2 and 1:4 solvents.

The phosphate elution peaks did not directly coincide with radioactivity in any of the column chromatographs.

Lipid solvent extractions on freeze-dried preparations

Two methods of extraction were used on the freeze-dried preparations :-

(a) An isopropanol-methanol 11:7 v/v extraction.

(b) A methanol-diethyl ether 1:1 v/v extraction followed by a series of solvent extractions on the methanol-ether extracted lipids according to the method of Handa (1963) for the isolation of the erythrocyte glycolipids.

The radioactivity elution patterns of the silicic acid/Hyflo columns of aqueous and dry isopropanol-chloroform extractions were very similar, varying only in the actual quantities of radioactivity eluted in the C-M elution solvents.

The total quantity of radioactivity recovered in the methanol-ether extracts was of the same order as that recovered in the isopropanol-chloroform extracts, but the former was sub-divided into acetone, diethyl ether and pyridine soluble fractions.

The silicic acid columns of the three extracts had the following regions of radioactivity :-

Extract	C-M 4:1 elution solvent	C-M 3:2 elution solvent	C-M 1:4 elution solvent
Acetone	-	large peak	-
Diethyl ether	-	large peak	tail end of C-M 3:2 peak
Pyridine	small peak	small peak	large peak

On one occasion, when dimethyl formamide was used as the last extraction solvent and not pyridine, the quantity of radioactivity extracted by the dimethyl formamide was less than by the pyridine, but the silicic acid-column chromatogram had only one sharp peak of radioactivity in the C-M 1:4 elution solvent.

As with lipid extraction from aqueous preparations, there did not appear to be any correlation between the phosphate and radioactivity elution patterns.

Thin-layer chromatography of the column elution fractions

a. The C-M-H_2O 80:30:3 solvent system

Radioactive fractions eluted by C-M 4:1, 3:2 and 1:4, when chromatographed in the C-M-H_2O, gave radioactivity only at the origin, Rf value 0.05 - 0.1. A sample of stock ^{14}C glucose also chromatographed to give radioactivity at this Rf value. The plates, developed in a saturated iodine tank, showed that all the samples were heterogeneous but the fractions from aqueous extracts were more heterogeneous than those from the same column eluent of a freeze-dried extract.

The material eluted by C-M 4:1 contained material chromatographically similar to the phosphatidyl ethanolamine standard. The spot also gave a positive ninhydrin reaction.

The material from the C-M 1:4 column elution solvent had a lipid spot in the iodine tank of Rf value 0.0 - 0.1. Unfortunately, a more positive analysis of the thin-layer plates was prevented by the presence of impurities in the

lipid standards, each of which resolved into four or more components on thin-layer plates.

There were three possible explanations for the presence of all the radioactivity at the origin :-

(a) Only free glucose was present in the original column elution fractions.

(b) Any lipid-glucose complex material present in the column elution fractions had been dissociated by the presence of the water in the thin-layer chromatography solvent.

(c) The lipid-glucose complex material had remained at the origin in the C-M-H_2O solvent system.

b. <u>The dimethyl formamide solvent system</u>

In contrast to the previous solvent system, the material in the C-M 1:4 elution fractions chromatographed in the dimethyl formamide solvent to give radioactivity of Rf value 0.8 - 0.85. A stock ^{14}C glucose sample gave radioactivity at the origin. The material in the C-M 3:2 elution fractions gave radioactivity at the origin and, on occasions, small peaks were present at Rf value 0.8 - 0.85. The dimethyl formamide solvent system was used mainly in conjunction with the column chromatographs of pyridine, ether and acetone extracts. Fractions from the acetone columns gave radioactivity only at the origin. Small quantities of radioactivity of Rf value 0.85 were occasionally present in the elution fractions of the ether extracts, but most of the radioactivity was at the origin. Fractions from the pyridine extracts in the 1:4 eluent gave a large region of radioactivity at Rf value 0.85.

The dimethyl formamide suppressed iodine saturation of the lipids and other colour detection reagents, but the material was visible under ultraviolet light at Rf 0.85. Also sulphuric acid charring of the plates gave a diffuse spot at this Rf value.

This radioactive material was not the result of metabolic incorporation of glucose, since shaking the material with ether and water and rechromatographing the two phases resulted in all the radioactivity appearing at the origin of the water sample and in a spot of Rf value 0.85, visible under ultraviolet light, appearing in the ether sample. This result also indicated that the association between the lipid and the glucose was of a loose nature, capable of dissociating in a highly polar solvent. These bonds may well be hydrogen bonds, which would explain any dissociation of this material in the C-M-H_2O solvent system.

Although in control experiments column chromatography resulted in elution of radioactivity in the C-M 3:2 with some overlapping into the C-M 1:4, subsequent thin-layer chromatograms developed in dimethyl formamide gave radioactivity only at Rf 0.05 - 0.1. The dimethyl formamide was chosen for its high dielectric constant, but it does not promote ionisation. Although several of the lipid standards moved on thin-layer plates developed in dimethyl formamide, e.g., cerebroside to Rf 0.7 - 0.8 and sphingomyelin to Rf 0.55 - 0.6, the resolution of lipids was incomplete.

Comparison of the material from the C-M 1:4 eluent with the material resulting from the triphosphoinositide incubation with ^{14}C glucose.

The material eluted from the columns in C-M 1:4 had the following properties in common with a component in the triphosphoinositide-glucose preparation :-

(a) In the C-M-H_2O solvent system the radioactivity and an iodine-staining spot were at the origin.

(b) In the dimethyl formamide solvent the radioactivity migrated with Rf 0.8 - 0.85, and this region fluoresced under ultraviolet light and charred with sulphuric acid.

(c) Shaking the material up with water and ether resulted in all the radioactivity passing into the water phase and

all the lipid material into the ether phase.

(d) Thin-layer chromatography of the material in a chloroform-methanol I:2 solvent and sulphuric acid charring of the developed plate gave a spot of Rf value 0.7-0.8 which passed on charring through several colour changes. The spot charred from blue to grey and finally to black. The radioactivity also migrated with this Rf value. Though the lipid standards were impure and on thin-layer chromatography gave several spots, none of them had a component which exactly matched this Rf value and charring properties.

(e) Carbohydrate estimations (using the anthrone reagent) on the eluent fractions of silicic acid columns of the triphosphoinositide and pyridine extracts showed there was a carbohydrate peak eluted in the C-M I:4 eluent of both chromatograms. This was present even when ^{14}C glucose without carrier glucose was used and when the chemical amount of added sugar were insignificant.

The similarities between the triphosphoinositide and the pyridine extracts suggested several possibilities, as follows:-

(i) The material is triphosphoinositide which is extracted along with glycolipids into the pyridine extract.

(ii) The material is a glycolipid which is also extracted as an impurity in the preparation of the triphosphoinositide from rat brain.

(iii) The material is an impurity common to both ghost extracts and triphosphoinositide preparation.

i. The evidence in support of the material being triphosphoinositide rests largely on the fact that the rat brains were extracted specifically for this lipid. The approximate concentration of inositol present (Dr. D.A. Nixon) in the triphosphoinositide preparation suggested that the material was 48°/o by weight triphosphoinositide.

Hokin and Hokin (1964) demonstrated the presence of triphosphoinositide in the erythrocyte membrane. Dittmer and Dawson (1961) found that both the free acid and the sodium salt were insoluble in acetone and ether, but the free acid was completely soluble in ethanol and methanol. If the triphosphoinositide were extracted from the ghosts by the methanol-ether 1:1, it would subsequently be extracted into the pyridine from the dried M-E extract.

There was only a faint indication of inositol in the C-M 1:4 eluent fractions of the silicic acid column of the pyridine extract, but the quantity of lipid associated with the radioactivity eluted in these fractions would be very low and the corresponding inositol concentration probably below detection.

It was not possible to get a positive stain for phosphate for the triphosphoinositide or pyridine extract on thin-layer chromatograms. But since none of the available phosphate reagents gave a satisfactory specific reaction with the lipid standards, this result was not considered to be valid.

The mobility of triphosphoinositide in the C-M 1:2 solvent system was questionable since Dr. Dawson was of the opinion that it would be stationary in this solvent system. However, a strong spot of Rf 0.7 - 0.8 was detected by sulphuric acid charring of a sample of triphosphoinositide donated by Dr. Dawson, when similarly developed on a thin-layer plate.

ii. The series of solvent extractions performed on the methanol-ether extract of ghosts was such as to isolate the glyco lipids into the pyridine solvent. Hexose associated with lipids was present in Fractions 36-39 eluted from silicic acid columns of both the triphosphoinositide and red cell ghost extracts. In the latter this directly coincided with the radioactivity of the complex material. The anthrone reagent which was used in the hexose estimations was found to

have no colour reaction with a series of standard inositol solutions.

A spot with mobility of the glycolipids was present in thin-layer chromatograms of the triphosphoinositide and pyridine soluble ghost extracts when run in a solvent system of C-M-H_2O 60:35:8 v/v (Yamakawa et al, 1963).

Yokoyama and Yamakawa (1964) isolated crude glycolipids from bovine brain. It is thus quite likely that glycolipids were extracted from the rat brains in the preparation of the triphosphoinositide.

iii. It was not conclusively proved that either glycolipid or triphosphoinositide was associated with the complex radioactivity, and the possibility of the involvement of a different lipid, present in both brain and erythrocyte membranes, could not be excluded.

The radioactivity eluted by C-M 4:1

On occasions, small quantities of radioactivity were present in fractions eluted by C-M 4:1, particularly in the chromatograms of the isopropanol-chloroform 11:7 extracts. Phosphatidyl ethanolamine was present in the thin-layer chromatograms of the material (Remfry 1965), as well as other unidentifiable material, and it was not possible to determine with which lipid spots, if any, the radioactivity had been associated. The possibility exists that the lipid associated with glucose was the same as the lipid eluted in the C-M 1:4 eluent, but in a different form, i.e., the acid and salt forms of the lipid. Rathbone (1962) recorded that phosphatidyl serine was eluted from a silicic acid/Hyflo column according to the acid and salt forms, in the C-M 4:1 and 3:2 solvents respectively. The low quantity of material eluted in C-M 4:1 prevented any further analysis, and a technique for increasing the yield and stability of this fraction would be desirable.

Increased glucose concentration in the incubation media

The results suggested that the relationship between increased glucose concentration and the concentration of the complex material isolated from the pyridine extract was a linear one. This does not agree with the saturation kinetics of the erythrocyte sugar carrier system. However, the estimated concentration of the complex material present in the pyridine extracts did not allow for the following sources of error :-

(a) The losses of lipid and radioactivity during the experimental procedures.
(b) Not all the radioactivity in the pyridine extracts was complex material, particularly at the higher glucose concentrations when there was overspill of free glucose into the pyridine.
(c) The radioactivity eluted in C-M 3:2, although mainly free glucose, sometimes contained small quantities of complex material.

The effects of inhibitors

Irreversible inhibitors

Dinitrofluorobenzene (DNFB) and mercuric ions both decreased the radioactivity extracted into the pyridine. The radioactivity that was extracted into the pyridine solvent of the DNFB-inhibited cells was eluted from the silicic acid column mainly in the C-M 3:2 solvent, but a small quantity was present in the C-M 1:4 eluent. The ether extract of the cells was coloured with the characteristic yellow of dinitrophenol, which is the product of DNFB hydrolysis. The radioactivity in the ether extract was eluted from a silicic acid column mainly in the C-M 4:1 and 3:2, but there was also a very small sharp peak in Fractions 36-38. The dinitrophenol was eluted in Fractions 18-23 in C-M 4:1. Thin-layer chromatography of these fractions showed they were heterogeneous, although there was a predominance of ethanolamine present.

It was not clear why the radioactivity was eluted at this earlier stage. The radioactivity in these fractions had two Rf values on thin-layer plates developed in dimethyl formamide. One was at the origin and was probably free glucose, the other was at Rf 0.40 which did not correspond to free glucose or to the complex material of the C-M 1:4 eluent. This material fluoresced under ultraviolet light and was faintly yellow in daylight, but it was not possible to make a more specific analysis.

The small quantity of material eluted in the C-M 1:4 ran in dimethyl formamide and had radioactivity at Rf 0.8 - 0.85, and this region also fluoresced under ultraviolet light. Thus a small quantity of complex material was present in the DNFB-treated ghosts, but it appeared likely that lipids which had reacted to form DNP-derivatives had different elution properties. Their nature could not be analysed further.

When the cells were inhibited by the mercuric ions, the quantity of radioactivity extracted by the pyridine was almost negligible and column chromatography of the extract gave only a small quantity of radioactivity in the C-M 3:2 eluent. Thin-layer chromatography of this material gave radioactivity mainly at the origin.

When radioactive mercury was used with non-radioactive glucose very little radioactivity was extracted into the methanol-ether solvent, but over 80% of the radioactivity that was extracted, was taken up by the pyridine solvent. The radioactivity of the pyridine extract was eluted from the silicic acid column as a sharp peak in Fractions 36-39 of the C-M 1:4 eluent. There was insufficient radioactive mercury in these fractions for scanning on thin-layer plates, but sulphuric acid charring of plates developed in C-M-H_2O 80:30:3, C-M 1:2 and dimethyl formamide solvents showed the lipid that was normally associated with the complex glucose was present.

Reversible Inhibitors

The results from the experiments with reversible inhibitors were inconclusive. Phloretin did not affect the elution pattern of radioactivity from the silicic acid column and ^{14}C stilboestrol did not appear to be associated with any particular lipid eluted from the column. However, only the butanol and acid-butanol extractions of the earlier experiments were used in these experiments.

It is not known whether the complex material eluted in C-M 1:4 from the pyridine-soluble extract from ghosts and from the triphosphoinositide preparation is involved in the transport of glucose across cell membranes. It appears that glucose can associate with a particular lipid component and such associations are of interest from a purely biochemical standpoint since this property is one involving only a very small fraction of the total cell lipids. The characterisation of the reaction, the discovery of the nature of the lipid and its possible physiological role offer important opportunities for future work.

The possible relevance to the findings of other workers may also merit further study, for instance, comparisons have been drawn by many workers between the sugar transport systems of certain bacterial strains and the red cell. Recently Kennedy et al (1966) claim to have identified the carrier or permease system of Escherichia coli. On the basis of the chemical properties of those reagents which they found inhibited the galactoside transport system, they determined the carrier to be protein, which they called the M protein. They have not isolated the M protein as yet, but claim that it is localised in the membrane of the E.coli to which it is held up by lipid-protein bonds.

Stein et al (1966) using an ingenious technique of loading DEAE-cellulose and "Celite" columns with erythrocyte

stroma and recording the differential elution of glucose and sorbose from the columns, have isolated and solubilised a glucose-binding component from the stroma. They have not discovered the nature of the component or established its involvement in glucose transport across the membrane, but they have demonstrated the existence of glucose-binding components within the erythrocyte membrane.

SUMMARY

1. A brief history is given of the development of the theory of the structure of the cell membrane and in particular of the red cell membrane, together with the history of the evidence for the existence of a facilitated sugar transfer system, differing from diffusion and probably involving the reversible combination of glucose with a membrane component.

2. In this work red cells or red cell ghosts were incubated with ^{14}C glucose for short intervals near $0^{o}C$ and evidence sought for any association of glucose labelling with membrane lipids. ^{14}C labelling was extracted by all the lipid extraction solvents used but only some of the extracts, when chromatographed on silicic acid columns gave reasonable quantities of labelling in elution fractions differing from those for free sugar. Both isopropanol:chloroform, 11:7, and methanol:ether, 1:1, when used to extract freeze-dried preparations, extracted radioactivity some of which was eluted from silicic acid columns in regions distinct from free glucose.

3. Thin-layer chromatography could not resolve the labelling from free glucose when the plates were developed in C:M:H_2O, 80:30:3, but material with a different migration from free glucose was obtained when dimethyl formamide was used as the developer. In this solvent the Rf was 0.8 - 0.85 and a spot of similar Rf value was visible under ultraviolet light. This material was chiefly present in the pyridine soluble fraction when the ghosts were extracted by the method described by Handa (1963).

4. Some of the radioactivity eluted from the silicic acid columns appeared to be associated with the elution of phosphatidyl ethanolamine but the material with an Rf value 0.85 in the dimethyl formamide was chiefly eluted in the C:M, 1:4, in association with the elution of glycolipids.

5. The lipid component associated with glucose in these ghost extracts appeared to be present in a triphosphoinositide sample prepared from rat brains.

6. Increasing glucose concentration of the erythrocyte incubation media did not appear to saturate the lipid component in the range of experiments carried out but dinitrofluorobenzene (DNFB) and Hg^{++} ions, inhibitors of the facilitated transfer in intact cells, greatly reduced the quantity of radioactivity extracted in association with lipids in the Pyridine soluble fractions. A small proportion of complex material, detectable by thin-layer chromatography, was still present in the DNFB experiment but none was present in the Hg^{++} experiment. Preliminary experiments with radioactive Hg^{++} showed some mercury to be associated with material eluted in the same region as the 'complex' in ^{14}C Glucose experiments but quantitatively the results were inconclusive.

9. In comparitive studies sheep erythrocyte extracts gave results somewhat similar to human erythrocytes but the pyridine soluble labelling was eluted in the region of free glucose and did not contain material which migrated to Rf 0.85 when chromatographed on thin-layer plates developed with dimethyl formamide.

10. Experiments were also carried out with competitive inhibitors of glucose transfer, stilboestrol, phloretin and phenolphthalein and with the irreversible inhibitor Triton X-100. None of these proved of special value since their actions included non-specific lipid affinities.

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