STUDIES OF RED CELL AND OTHER MEMBRANE EXTRACTS IN RELATION TO GLUCOSE PERMEABILITY

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ABSTRACT

Two possible mechanisms involved in the facilitated transfer of glucose through red cell and other membranes have been investigated.

In the first section, the hypothesis that glucose transfer involves phosphorylation was tested by estimating phosphatase activities in human red cells, adult and foetal guinea-pig red cells and kidney homogenates, and the effects upon them of known inhibitors of glucose transfer. No correlation between phosphatase activity and glucose transfer in the red cell could be found. Histochemical studies are presented to show the distribution of acid and alkaline phosphatases in the adult and foetal guinea-pig kidney, and the placenta.

In the second section, the possibility is explored that glucose forms a reversible complex with a phospholipid component of the cell membrane. Methods were developed to extract phospholipids from sheep placental tissue and human and sheep red cells, to separate them chromatographically and to isolate the glucose-lipid complex.

Uptake of the irreversible inhibitor 2,4-dinitrofluorobenzene (DNFB) labelled with [^{14}C] was measured, and calculated to be 400 million molecules per red cell to give full inhibition of glucose transfer. The lipids extracted from the DNFB-inhibited red cells contained radioactivity equivalent to 10-20 million molecules DNFB per red cell, which considerably exceeds the number of carrier sites thought to be involved in glucose transfer. A large proportion of the radioactivity was found in the cephalin fractions, and the possibility is discussed that phosphatidyl-ethanolamine is the membrane carrier component.

The technique of monolayers was used to study the surface behaviour of certain phospholipids in the presence of glucose, and the preliminary results obtained were discussed.

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CHAPTER 1.

INTRODUCTION

General

The cell membrane is a diffusion barrier between the external and internal environment of the cell. This concept, fundamental to the whole of the modern study of physiology, has developed on the heels of Bernard's "la fixite du milieu interieur": thus the idea of the whole animal becoming independent of its external environment by maintaining a constancy of its internal composition has been expanded to the idea of each cell individually regulating its own composition by selective permeability of its membrane to the constituents of its surrounding tissue fluids. However, the result of the regulation is not necessarily "fixite" since a cell is in a dynamic state so long as it is alive and its function may depend on alterations in composition, as, for instance, in the uptake of oxygen by red cells, involving changes in pH and electrolyte composition.

The cell theory of life was formulated by Schwann & Schlieden in about 1840. Soon afterwards it was recognised that, to explain the osmotic effects observed in both plant and animal cells, the cells must be bounded by a semi-permeable membrane, that is a membrane permeable to water but only slightly permeable to sugars and salts. This permeability barrier was known as the plasma membrane and was realised to be very thin and delicate, quite different from the rigid cellulose cell wall of plants, for instance, or the tough outer membrane of sea-urchin eggs, both of which are freely permeable to both water and salts.

In 1895 Overton, working with plant cells and later with frog muscle, obtained the first evidence on the nature of the plasma membrane. He showed that the membrane, although almost impermeable to salts and sugars, is freely permeable to alcohols, aldehydes and alkaloids, which are more soluble in fat solvents than they are in water. He deduced that the plasma membrane is lipoid in character, and predicted correctly that major constituents are cholesterol and lecithin.

In 1897 Hedin came to a similar conclusion using ox erythrocytes, and formulated the rule that, with polyhydric alcohols, the rate of penetration decreases with the number of -OH groups. He also noted that urea and glycerol, although lipoid-insoluble, have high penetration rates.

Other discrepancies were observed using human erythrocytes. For instance, although they were permeable to glucose, they were not permeable to the isomeric inositol. Another unexplained feature was the high concentration of potassium ions in erythrocytes, maintained there against a considerable concentration gradient.

Theories have been put forward to explain these and other anomalous properties of the cell membrane, and in fact the nature of the permeability of membranes to salts and sugars has been one of the most widely explored problems of the last twenty years. Electron microscopy has been used to study the minute structure of membranes, kinetic methods have been used to measure the rates of penetration of substances through the membrane and enzymatic studies to identify the energy-sources, where these are necessary. Another line has been the isolation of components

of the membrane, in order to identify them and attempt to find which are directly concerned with the mechanisms of permeability. This thesis will describe the methods used to study the components thought to be concerned with the permeability of red cells and other membranes to glucose. First, however, the possible mechanisms of permeability will be discussed.

Transport Mechanisms

Many examples could be given of special transport mechanisms postulated to explain the permeability properties of a particular cell membrane, but three will be described here to illustrate some of the problems involved and the variety of possible explanations: (1) the uptake of glucose by the small intestine and proximal tubule of the kidney, (2) removal of sodium ions from erythrocytes, (3) penetration or loss of glucose through the erythrocyte membrane.

(1) Uptake of glucose by gut and kidney.

The absorption of glucose from the small intestine by the luminal cells of the mucosa is a process which takes place even when the concentration of glucose in the small intestine is lower than that in the blood. In 1925 Cori showed, using an intact animal, that pentose sugars were absorbed more slowly than hexoses and that the rate of absorption of glucose is not proportional to the concentration in the lumen but reaches a maximum at a certain concentration - that is, the process shows a saturation effect. He also showed, in 1926, that there is competition between different sugars for the absorption

mechanism. More quantitative results were obtained by Fisher and Parsons (1949, 1950, 1953). They were able to show that oxygen was required for the active uptake of glucose, that the jejunum was able to take up glucose more rapidly than other parts of the small intestine, and that the rate of uptake was related to the initial luminal concentration by an equation of the Michaelis-Menten type.

Thus the absorption of glucose from the gut shows the distinctive features of an "active transport" system: diffusion against a concentration gradient, the requirement of an energy supply implied from the need for oxygen, difference of rate between sugars of slightly different configuration, competition between sugars and saturation of the absorption mechanism.

Several similar features are shown by the reabsorption of glucose in the kidney. Cushny (1917) showed that it was possible to explain the disappearance of glucose from the glomerular filtrate by an active reabsorption of the glucose back into the blood by the kidney tubular cells. This led to the idea of glucose being a "threshold substance"; that is, if the concentration in the glomerular filtrate is too high, the reabsorptive mechanism becomes saturated and the excess is lost in the urine, giving glycosuria. The micropuncture technique, demonstrated by Richards (1925) in amphibia, was developed for mammalian kidney and it was eventually shown that the sole site of glucose reabsorption was the proximal convoluted tubule (Walker et al., 1941).

The cells of the proximal tubules, like most of those in the small intestine epithelium, are distinguished by their "brush borders", which are actually microvilli, on the luminal edge, and the cells are tightly packed with mitochondria, which would be the source of much energy if

required. The process can be completely blocked by phlorrhizin (Von Mering, 1899) in a dose of 200mg./Kg. This effect is specific to sugars and does not affect the other substances absorbed in the proximal tubule, for instance amino-acids and phosphate.

Glucose is normally reabsorbed completely, but sugars with slightly different configurations behave differently. Xylose, fructose and galactose are reabsorbed against the concentration gradient, but the process is slower and not complete. They behave as if competing with glucose for the active process and, in fact, the reabsorption of xylose can be almost completely inhibited by saturating the mechanism with glucose.

The sites of absorption have a high concentration of alkaline phosphatase activity in the brush borders. This observation gave rise to the phosphorylation theory of Wilbrandt and Laszt (1933) which was offered as an explanation of the fact that both glucose absorption and certain phosphorylating reactions were inhibited by mono-iodoacetate. According to this theory, glucose was phosphorylated on the luminal side of the cell by hexokinase (Drabkin, 1948), it travelled across the cell in this form and was then dephosphorylated by glucose-6-phosphatase. Although tempting, since all the enzymes are there, it does not explain how the lipid-insoluble glucose-6-phosphate crosses the lipid membrane. Sols (1956) was able to show that hexokinase phosphorylated galactose and 3-methyl-glucose more slowly than the rate at which they were actively transported, and this seemed to eliminate hexokinase and glucose-6-phosphate from the scheme. There was a slight possibility that the glucose-phosphate bond might be in the 1-position, but this was eliminated by Crane and Krane (1956) who showed that 1-5-anhydro-D-glucitol, which cannot be phosphorylated

in the 1-position, is nevertheless transferred against a concentration gradient. In the kidney, it is known that patients with Von Gierke's disease, with no glucose-6-phosphatase in their proximal tubules, do not suffer from glycosuria, so presumably their glucose reabsorption mechanism is intact. This, and other evidence, has discredited the phosphorylation theory but has done little to suggest an alternative role for the phosphatases whose distribution in the body correlates so well with that of active glucose transfer.

(2) Sodium extrusion from red cells.

In blood which has been stored for some time at $4^{o}C$, sodium ions are equally distributed between red cells and plasma, and so are potassium ions. If the blood is then incubated at 37^{o} in the presence of glucose, it is found that sodium ions are extruded from the red cells and that potassium ions enter the red cells. The result of this is a considerable concentration gradient between red cells and plasma of both Na^{+} and K^{+}, though in opposite directions, but only a small electrical potential difference. In 1941 Dean put forward the hypothesis of the "sodium pump" to explain this phenomenon. In 1956 Glynn proposed that, since the entry of K^{+} into the red cells followed Michaelis-Menten rather than straightforward diffusion kinetics, the inward flow of K^{+} as well as the outward flow of Na^{+} was achieved by an active mechanism.

The transport of Na^{+} and K^{+} in the red cell shows typical features of an active mechanism: movement of the ions against an electro-chemical gradient, dependence on energy supply: saturation-type kinetics,

competition between sodium and lithium for the exit mechanism and between potassium and rubidium for the entry mechanism, and specific inhibition by the cardiac glycosides.

Many of these features are best explained by a transport system whose rate-limiting step is the formation of a carrier complex. There could be either two types of carrier, one for sodium (for which lithium competes) and one for potassium (for which rubidium, but not lithium, competes), as suggested by Solomon (1952), or one type of carrier which has a sodium affinity on the inside of the membrane and a potassium affinity on the outside (Shaw, 1954). The second scheme offers the better explanation of the apparent linkage between Na^+ extrusion and K^+ accumulation observed in erythrocytes and other cells.

Suggestions have been made as to the nature of the carrier. It seems likely that the phospholipids, which make up a large proportion of the membrane material, could be involved in mechanisms for the transport of electrolytes, since their polar groups are on the outside of the membrane and could attract the charged ions. Solomon et al (1956) studied various phosphatidyl fractions and found that the fraction containing phosphatidyl serine had the greatest ability to discriminate between Na^+ and K^+. Phosphatidyl serine was actually suggested as a possible Na^+ carrier by Kirschner in 1958, as a result of work on the cation content of phospholipids from swine erythrocytes.

It is not clear whether the carrier complex system would require energy, for instance to convert the sodium affinity into potassium affinity in the system suggested by Shaw, or whether its activity could be accounted for by thermal diffusion. Certainly energy is required at some stage: in nerve and muscle this is derived from oxidative metabolism via the Krebs cycle, but in red cells, where the enzymes for the Krebs cycle do not exist, it is derived from glycolysis. Both metabolic systems produce high-energy phosphate in the form of ATP.

Clarkson & Maizels (1952) were the first to show that human red cell ghosts, well washed, had a high ATPase activity, splitting off inorganic phosphate from ATP added to the ghosts and leaving ADP. Both ATPase activity and sodium transport require the presence of potassium as well as of sodium. Schatzmann (1953) showed that cardiac glycosides inhibit the reaccumulation of potassium by red cells after cold storage without affecting glucose utilisation. From this and further evidence, Dunham & Glynn (1961) concluded that, in the red cell, ATPase activity and active transport are correlated, and Glynn (1962) showed that for each molecule of ATP split three sodium ions are transported.

(3) Glucose transfer in red cells.

The first comparative study of glucose permeability in red cells was by Kozawa in 1914. He used a haemolysis technique to show that human and monkey erythrocytes were permeable to both hexose and pentose

sugars. Dog red cells, he found, were only slowly permeable, and those of all other animals studied were impermeable. At the present time, it would be unusual to assert that the red cell of any species is actually impermeable to sugars. Modern physical techniques still cannot detect any uptake of glucose in most species, but chemical analysis of glucose in plasma and red cells of, for instance, the guinea-pig shows an uptake by the red cells following an increased plasma level, which may take several hours.

Accurate measurements of penetration rates in the human erythrocyte were made possible by the technique developed by Ørskov (1935). This method depends on the fact that glucose penetrates the red cells much more slowly than water, so the red cells may be assumed to be always in a state of osmotic equilibrium. When red cells are suspended in isotonic saline and then sugar dissolved in isotonic saline is added, the cells are observed to shrink osmotically, then to swell to their original volume as the sugar penetrates the cells. A light beam is passed through the suspension and the changes in light intensity, measured photoelectrically, depend on the volume changes of the cells.

Using this method and an indirect method based on changed cell fragility at different cell volumes, Wilbrandt, Guensberg & Lauener (1947) showed that the rate of penetration of glucose into human erythrocytes was not linearly dependent on the concentration difference, but fell off at higher concentrations. LeFevre (1948) confirmed that the process was not one of simple diffusion and showed that it could best be explained on the basis of a "carrier system" involving the temporary formation of a complex with some constituent of the cell membrane.

The first evidence that the primates were not alone in possessing glucose-permeable red cells was produced by Goodwin (1954, 1956) and by Widdas (1953b, 1955). Goodwin showed that the proportion of the blood glucose present in the red cells, while low in the adult of most species, is very much higher in the foetal and neonatal animal, and in the foetal red cells of rabbit and guinea-pig is, in fact, equal to the plasma glucose concentration.

Widdas investigated the rates of glucose penetration into foetal red cells and found that in sheep, deer, pig, rabbit and guinea-pig penetration is fairly rapid. They compared quite closely with the penetration rate in human erythrocytes, and the foetal guinea-pig red cell had a penetration rate more rapid than that of the foetal human red cell.

After birth the rate of penetration falls; in the guinea-pig it was measurable for 9 days and detectable up to the 51st day. Although it is possible that the red cells lost their carrier components during the post-natal period, it is more probable that the foetal red cells were gradually replaced by adult ones with fewer carriers, or capable only of simple diffusion.

Widdas also showed that neither the adult nor the foetal cat erythrocyte was detectably permeable to glucose. Perhaps this is why cat erythrocytes have a low potassium level, because glucose would not be present in sufficient amounts to provide energy for the sodium pump, or perhaps glucose permeability and sodium transport are so closely linked and inter-dependent that one cannot operate without the other.

In the case of guinea-pigs and other animals with adult rates too slow to measure, glucose probably does penetrate fast enough to provide energy for the sodium pump. It was pointed out by Widdas (1954) that the inward transfer of glucose in the human red cell is about 250 times the rate which would be necessary for the cell's metabolic requirements, and that a rate which would just fulfil the requirements would probably not be measurable. This was supported by an experiment of Morgan, Kalman, Post & Park (1955) which showed that in rabbit red cells, which normally appear impermeable to glucose, glucose may be observed to enter and accumulate if glucose utilisation is first inhibited. The original observation of Kozawa thus remains true to the extent that, amongst adult animals, only the primates retain a rate of glucose penetration far in excess of the apparent metabolic requirement. It confers no obvious advantage upon the animal, except to almost double the glucose-carrying capacity of the blood.

Human erythrocytes have been used most to study the characteristics of glucose penetration. There are two main differences from the other systems discussed previously. First, the glucose transfer is normally always down the concentration gradient. Secondly, the transfer is not "active", that is, metabolic energy is not required: inhibitors of the glycolytic pathway such as iodoacetate do not affect glucose transfer (LeFevre, 1948).

Many of the other characteristics of facilitated diffusion are similar to those of active transport. In particular, there is the saturation of the mechanism at high concentrations, specificity of the mechanism - aldose sugars such as glucose penetrate more rapidly than ~~hexose~~ ketose sugars such as sorbose (LeFevre & Davies, 1951); competition between sugars; and specific competitive inhibition by phloretin (Wilbrandt, 1950).

Using the method of Ørskov to measure the diffusion rates both into and out of red cells, it was possible to derive results which could be compared with the theoretical results to be expected from certain model systems.

The model system used by LeFevre & LeFevre (1952) assumed that the sugar molecule exists in a free state outside and inside the red cell, but to cross the membrane it forms a reversible association with a carrier molecule. The sugar-carrier complex then moves or reorientates itself across the membrane. In this system the rate of penetration would depend on the number of carrier molecules available, the dissociation constants of the complex at each interface, and the concentration gradient of the complex across the membrane. They assumed the rate-limiting step to be the dissociation of the complex on the inside of the membrane. This model agrees with the experimental facts in most respects, except at high concentrations of glucose when it was necessary to make the additional postulate that the glucose was acting as an inhibitor of its own transfer. LeFevre has since dropped this model in favour of a similar one nearer to that of Widdas.

The model used by Widdas (1952) was derived from the adsorption equilibrium equation for interfaces, and in its simplest form is

$$\text{Transfer Rate} = a\frac{[P]_1}{[P]_1 + K} - a\frac{[P]_2}{[P]_2 + K}$$

where a = constant, depending on the characteristics of the membrane, and the number of carrier molecules.

$[P]_1$ & $[P]_2$ = concentration of hexose outside and inside the red cell.

K = dissociation constant of the complex.

The same expression could be derived from the Michaelis-Menten equation for the fraction of enzyme combined with substrate, so it would be valid whether the carrier complex were physical or chemical.

This model system assumes that there is a carrier which adsorbs hexose and which is in equilibrium with hexose at each interface, and that the carriers pass backwards and forwards across the membrane whether or not they are saturated with hexose. It was presumed that the rate-limiting step would be the relatively slow movement of carriers and complexes between one interface and another. The rate of transfer would then depend on the fraction of carrier saturated with hexose at the two interfaces, which in turn is influenced by the affinity of the sugar for the carrier. If the affinity were high (as in the case of glucose), the dissociation constant K would be small and the transfer would show saturation at a fairly low sugar level. If the affinity were low, however, e.g. sorbose, then K would be large and the kinetics would not differ much from those of free diffusion. This agrees with the experimental facts; in fact it was found that where the affinity

is high the transfer becomes approximately dependent on the difference in the reciprocals of the hexose concentrations at the two sides of the membrane (Widdas, 1951).

Wilbrandt (1956) came to the same conclusion, using a similar model, and he expressed the rate of transfer as

$$V = V_{max} K_m \left(\frac{1}{S_2} - \frac{1}{S_1}\right)$$

where V = observed rate.

V_{max} = maximum rate - the "capacity factor".

K_m = the "affinity factor".

S_1 & S_2 = concentrations of sugar on the outside and inside of the red cell.

If this model were correct, then curious results would be expected when the rates of penetration of hexoses with different affinities are compared: at high concentrations the hexose with the higher affinity would penetrate more slowly and at low concentrations would penetrate more rapidly than the other. The experimental results agree with this prediction.

The dissociation constant of the sugar-carrier complex can also be expressed as the half-saturation constant. That is, the concentration of hexose at which half the carrier is saturated. Widdas (1954) found it to be about 10 mM for glucose at 37°C and pH 7·4; LeFevre (1954) found it to be 9 mM. By using a different technique based on glucose exits from red cells at 37°C, Sen and Widdas (1962a) obtained a value of about 4 mM over the pH range 5-8. Park et al (1956) obtained a value of 4 mM for rabbit red cells using a chemical technique. This suggests that the membrane component concerned is of a similar nature in the two species despite the difference in rates of glucose transfer.

This quantitative agreement between different workers in respect of parameters of transfer strengthened the view that glucose crosses the red cell membrane by a carrier mechanism. However, the kinetics do not necessarily imply that the loading and unloading of the carrier is unassisted and another possible mechanism should be discussed.

One of the earlier models of Rosenberg and Wilbrandt (1952) involved an enzyme reaction at each interface by which the sugar-carrier complex was formed and broken down. The enzymes were not specified, although phosphorylation was suggested. The idea was stated again slightly differently by Wilbrandt & Rosenberg (1961) by drawing a parallel between "transferase systems" and membrane carrier systems. It was pointed out that the co-enzyme of a transferase reaction acts in effect as a carrier molecule, and that if it were lipid-soluble it would be able to move across a lipid membrane between two enzyme molecules located on either side of the membrane. The transport system in bacterial membranes appears to be similar to this, and the enzymes or co-enzymes particularly concerned with transport have been called "permeases" (see Mitchell, 1957). Englesberg (1964) showed that for the permeation of glucose in bacteria there were energy-dependent and energy-independent components. In E. coli the galactoside transport system has been widely studied, and from the results obtained for the transport of glucose as well as galactose and other sugars Koch (1964) has derived a model which involves three stages: (i) a mobile carrier within the membrane which transports the sugar by facilitated diffusion, (ii) a permease on the outside of the membrane which catalyses the attachment of the sugar to the carrier and thus renders the transport process more rapid and specific, (iii) an active process involving high

energy bonds on the inside of the membrane responsible for 'against the gradient' accumulation. In this last model the action of permease does not require the expenditure of energy. If phosphatases were involved in glucose transfer in the red cell, it could be in the role of a permease. But as yet there is no evidence in favour of such a theory.

Another possibility is a theory based on the penetration of glucose through pores in the membrane. Although it is now realised that a cell membrane is not like a sieve and that speed of penetration does not depend primarily on molecular size, nevertheless the membrane of a living cell is in a state of thermal agitation and it is probable that temporary pores are continually being formed. If the middle layer of the membrane were entirely lipid and non-polar, as it appears in the formalised diagrams of cell membranes, then these pores would be impermeable to hydrated molecules such as sugars unless solubilised by attachment to a lipid-soluble carrier molecule. Since, however, water itself crosses the membrane very rapidly, there must be channels or pores where the presence of protein or of polar groups on the lipids confer hydrophilic properties which allow water, or possibly even larger hydrated molecules, to pass through.

Two theories based on the presence of pores in the membrane have been put forward: the polar pore hypothesis of Danielli (1954) and the polar "creep" hypothesis of Bowyer & Widdas (1956).

From a combined consideration of the mobile carrier system, the enzymatic carrier system and the polar pore system, Bowyer (1957) drew the following conclusions:-

(1) The carrier systems imply a mobile membrane component which could be lipid, lipo-protein, protein or contractile protein. If it were lipid, the system would involve re-orientation of the carrier molecule under thermal agitation so that the sugar molecules came to lie in the opposite interface. If it were protein, it would involve transfer of the protein through a lipid barrier, or a definite discontinuity in the lipid layer.

(2) The pore theory implies a non-mobile membrane component which could be protein, polysaccharide or nucleic acid. Alternatively, the pore could be temporary, with no special protein layer but with enough polar groups to facilitate the transfer.

In 1958, Bowyer & Widdas abandoned the polar pore theory when it was demonstrated by Park et al. (1956) and Rosenberg & Wilbrandt (1957) that glucose could be made to travel uphill if a second, competitive sugar was moving in the opposite direction down the gradient. This process, known as uphill transport by counterflow, could be explained only on the basis of a mobile carrier mechanism.

A variation of the theories of enzymes and pores is the dimer theory of Stein (1961). He postulated that an enzyme-like substance called a "dimerizer" at the cell membrane surface caused two molecules of glucose to form a dimer, thus losing some of their hydrophilic groups so that the dimer molecule then passed through a pore in the membrane with less hindrance.

One of the most notable features of glucose transfer, whether in the red cell, gut or kidney, is the specificity of the mechanism which

can distinguish between isomers. LeFevre & Marshall (1958) suggested that it was not the configuration of the molecule which mattered but its shape. Free hexoses and pentoses in aqueous solution can equilibrate in several ways, but the most strain-free, and therefore the most stable energetically, is a C1 pyranose ring in the "chair" rather than the "boat" form. The more stable the sugar in this form, the greater is its affinity for the carrier. Thus the reactive site on the cell surface must be able to distinguish between different geometric shapes.

For active transport in the gut, Crane & Krane (1959) found, by an isotope method, that the only requirement apart from D-configuration and the pyranose ring formation was a hydroxyl group at C2. They could not, however, show what reaction the C2 hydroxyl had to undergo during transport.

On the basis of evidence of this type, Rosenberg (1961) put forward the "multiple hydrogen bond theory". Since none of the hydroxyl groups at carbon atoms 1, 2, 3, 4 and 6 singly is necessary for equilibration of sugars (though C2 is essential for accumulation in gut), the affinity of the sugar for the carrier must be due to the combination of several -OH's, probably by the formation of hydrogen bonds between the sugar and suitable groups of the carrier.

There is another tissue in which glucose transfer has been studied, which should be briefly mentioned, and that is the placenta. Huggett, Warren & Warren (1951) suggested that simple diffusion would not account satisfactorily for the qualitative facts observed during placental glucose transfer from mother to foetus in the sheep. Widdas (1952)

analysed their quantitative results and showed them to be in reasonable agreement with the kinetics predicted from a membrane carrier system. He suggested that, although the total transfer involves physical diffusion of the sugar across relatively large distances as well as penetration of cell membranes, it is the membrane transfer which is the rate-limiting step. The deviation from diffusion kinetics is seen most clearly at high maternal blood sugar levels when the transfer mechanism becomes saturated.

It is interesting to note that placental tissue contains a fairly high level of phosphatase activity, although of course there are many active processes in the placenta with which this could be associated.

The Nature of the Carrier Component

To sum up: kinetic studies on the permeability of red cell membranes to glucose strongly favour the sugar-carrier complex hypothesis. In gut, kidney and placenta, the formation of such a complex could well be the rate-limiting first step by which the sugar penetrates the membrane to enter the cell. The kinetic studies do not distinguish between lipid, lipo-protein and protein as the carrier component, and so far no such carrier has been demonstrated.

If the carrier component were protein it would probably have enzymatic properties, even if its function in glucose transfer were not strictly enzymatic. A parallel to this would be actomyosin - the contractile protein from muscle, whose only apparent behaviour *in vitro* is that of an ATPase. Thus it was considered worth while to look again at the phosphatases, under conditions known to affect glucose transfer.

The first experimental section of this thesis will describe some of the properties of red cell phosphatases in the human and in the adult and foetal guinea-pig, and the effect on their activity of known inhibitors of glucose transfer. The distribution of phosphatases in the guinea-pig placenta and adult and foetal kidney will be demonstrated histochemically.

The second experimental section will be concerned with the possible role of lipids in glucose transfer. Interest has focused mainly on the phospholipids whose polar groups would be most obviously capable of hydrogen bonding with sugar molecules.

Before discussing the evidence for the involvement of lipids in transport mechanisms, the nature of the lipids themselves and their place in the cell membrane should be described.

Cell Membrane Phospholipids

Methods for the separation and identification of red cell lipids have been developed mainly by Hanahan et al. and methods of quantitative analysis by Dawson, and they have recently been reviewed by Marinetti (1962), Dittmer (1962) and Ansell & Hawthorne (1964).

Their work may be summarised very briefly as follows: in all species studied, 90-92% of the total blood lipid residues in the erythrocyte stroma or ghosts; of this lipid, about 30% is the "neutral lipid" - cholesterol with some glycerides and other non-phosphorus-containing, chloroform-soluble substances. The rest is made up mostly of the phosphatides - lipids which carry an electric charge due to the polarisation of either the phosphate group or a nitrogen-containing group, or both. There are two main types of phosphatides: the glycero-

phosphatides and the sphingophosphatides. The basic structural unit of the first is glycerophosphoric acid; when this is acylated with two long-chain fatty acids it gives phosphatidic acid (which may exist in the tissues in small amounts); the third component is usually a nitrogenous group which becomes esterified to the glycerophosphoric acid to form a phosphate diester. The nitrogenous group may be choline, ethanolamine or serine; the final molecule is then called phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine. They can exist in slightly different structural forms called plasmalogens. The strictly chemical nomenclature of these substances is not always used. In this thesis the suggestions of Dittmer (1962) will be followed, namely that the term "lecithin" should be used to cover phosphatidylcholine and choline plasmalogen, and "cephalin" to cover phosphatidylserine, phosphatidylethanolamine and their respective plasmalogens. Also that the enzymes which split off one or both of the acyl groups from the glycerophosphatides, which were formerly known as lecithinases, should be called phospholipases, and their hydrolysis products as lysophosphatidylcholine, etc.

Inositol-containing phosphatides are found in red cells. Their structure seems to be variable, but some, if not most, exist as phosphatidylinositol (Hanahan & Olley, 1958). Other groups which may replace choline, ethanolamine and serine to form diacylglycerophosphatides are other amino-acids, peptides and glycerol, though these are not necessarily present in red cells. Red cells contain small amounts of polyglycerophosphatide, which is probably the same as cardiolipin originally extracted from heart muscle by Pangborn (1947).

The sphingophosphatides have as their basic unit of structure the phosphate ester of sphingosine, to which is added another long-chain fatty acid and a nitrogenous base which is usually choline. This structure is known as sphingomyelin. Other substances related to this group, though not strictly phosphatides, are the cerebrosides. In these the phosphate and choline are missing and replaced by a sugar, usually galactose.

Lipids of all types are usually referred to in the plural, not only because of the possible variation in end-groups, but because there can be an enormous variety of fatty-acid chain lengths and degrees of unsaturation without altering the properties of the substance enough to justify giving it a new name.

The distribution of the various classes of phospholipids in the plasma and red cells of man and ox were compared by Hanahan, Watts & Pappajohn (1960). A more extensive survey was made by Dawson, Hemington & Lindsay (1960) in which they compared erythrocyte and erythrocyte ghost phospholipids in several species. They found that the main phospholipid constituents of human red cells were phosphatidyl-choline, phosphatidylethanolamine and sphingomyelin, in that order. Smaller quantities of phosphatidylserine, choline plasmalogen, ethanolamine plasmalogen, phosphatidylinositol, phosphatidic acid and serine plasmalogen were also present. In red cell ghosts there seemed to be more phosphatidylserine and less phosphatidylethanolamine, and slightly different proportions of plasmalogens.

. The pig and the horse were like the human in having phosphatidylcholine and phosphatidylethanolamine as the largest phospholipid components. The ox, sheep and goat, on the other hand, were rather low in these components, but had high sphingomyelin. The proportions of the other constituents remained about the same. This finding qualified the work of Turner (1957) who could find no lecithin at all in the red cell ghosts of ruminants. Polyglycerophosphatide was not measured in the human, but was present in small amounts in the whole cells and ghosts of all other species investigated.

An attempt to correlate the phosphatide content of the red cell with its permability properties in various species was made by de Gier & van Deenan (1961). They calculated the amount of lecithin present as a percentage of total phosphatide and found it to increase in the following order: sheep, ox, pig, human, rabbit, rat. They pointed out that permeability of the red cell to glycerol also increases in the same order, and that thus the amount of lecithin probably has some bearing on the permeability characteristics of the membrane. The relevance of this to glucose permeability is rather doubtfull since rabbit and rat have slower rates of glucose transfer than human red cells. This thesis will describe experiments to extract and separate lipids from human and sheep red cells, to compare their lipid composition and to see if there is a component present in greater amounts in human than in sheep which could correlate with the higher rate of glucose transfer in the human.

Placental Phospholipids

Analysis of the phospholipids of placenta have been performed mostly on the products of acid hydrolysis of the placental tissue. Determination of the phosphoric esters obtained have been performed by Porcellati, Curti & Luciani (1959) who found that, in human placenta, phosphorylethanolamine was the main constituent (50%), then glyceryl-phosphorylethanolamine (27%) and glycerylphosphorylcholine (18%). The remainder was phosphorylcholine and phosphopeptide. From acid hydrolysis of goat placenta, Neil (1961) was able to identify phosphoryl-ethanolamine and phosphorylcholine.

Recently (Hosoya & Fujimori, 1964) human placenta was extracted with lipid solvents and separated chromatographically by the method developed for red cells (Hanahan et al, 1957). The following phospho-lipids were identified: cardiolipin, phosphatidylserine, phosphatidyl-ethanolamine, phosphatidylinositol, phosphatidylcholine, lysophosphatidyl-choline and sphingomyelin, that being the order in which they were eluted from the silicic acid column. This thesis will describe the results obtained using a similar method to separate and identify sheep placental lipids.

Phospholipid-Sugar Complexes

When lipids are extracted from a tissue or membrane with an organic solvent, a certain amount of non-lipid material is extracted at the same time, such as amino-acids, salts, urea, sugars, water-soluble phosphate esters. The reason appears to be (Marinetti, 1962) that phosphatides are able to solubilize non-lipid material in organic

solvents, and may even form complexes, certainly with salts and possibly with sugars.

In the 19th century it was thought that a definite family of compounds was formed by reaction of phospholipids with sugars, and they were called "jecorines" (for instance, Mayer & Terroine, 1907). Little evidence has been produced for jecorines in recent times and a reversible complex is now considered more likely. LeFevre, Habich, Hess & Hudson (1964) have made lipid extracts of red cells and ghosts in the presence of $[^{14}]$C-glucose and found that, by extracting with hot ethanol-ether and then drying under vacuum at 50^{o} or even 95^{o}, an extract containing glucose up to 5×10^{8} molecules per ghost may be obtained. Lecithin or cephalin alone will take up roughly equivalent amounts, but olive oil will take up none. All monosaccharides tested behave like glucose, but the alcohol D-mannitol did not.

The mechanism appeared to be rather unspecific, with the forces involved rather weak. For instance, the glucose could be recovered by shaking the extract with successive amounts of water, and on paper chromatography dissociation of the complex was too great to identify any particular phosphatide with which it was associated. On thin-layer chromatography using non-aqueous developing solvents, however, some of the activity was recovered in the lecithin and lysolecithin spots.

The action of DNFB (dinitrofluorobenzene) was interesting: if the red cells were incubated with DNFB before adding glucose and extracting, the amount of glucose complex obtained was reduced to about one third.

This thesis will describe experiments, using several extraction techniques, under more physiological conditions and with more detailed

analysis of the distribution of radioactivity. Experiments will also be described on the uptake of DNFB by red cells and the effect of DNFB on glucose uptake.

Recently, Stein (1964) has shown that although DNFB is primarily a protein reagent, some is taken up by constituents which resist digestion by papain. He treated washed red cells with [14-C] and [3-H] - DNFB at different concentrations, measured the inhibition of sugar transfer, then haemolysed them and isolated the membrane fraction. The membrane material was treated with papain to digest the protein and yield peptides, then hydrolysed so that the non-peptide material was precipitated. He measured the amounts of [14-C] and [3-H] present in the peptide and precipitate, and calculated that the precipitate (papain resistant core) was more likely to contain the components active in glucose transport.

Phospholipid Metabolism

Apart from the few suggestions mentioned earlier in this introduction, the only phospholipid which has had a definite role postulated for it is phosphatidic acid. Hokin & Hokin (1963) and Hokin, Hokin & Mathison (1963) have produced evidence to show that the formation of phosphatidic acid from diglyceride and ATP, and its breakdown to diglyceride and phosphoric acid, that is the "phosphatidic acid cycle", would be in effect an ATPase system, and that it can take place in the red cell membrane fast enough to account for the breakdown of ATP which occurs to provide the energy for the sodium pump. However, it has not been possible to demonstrate the phosphatidic acid cycle and the sodium pump

simultaneously in the intact red cell, and it is possible that phosphatidic acid is only a step in the synthesis of other phospholipids.

Work on the metabolic synthesis of phospholipid in the red cell is rather limited and until recently it was thought that, since the red cell has fewer enzyme systems than nucleated cells, little synthetic activity took place. James, Lovelock & Webb (1959) suggested that some phospholipids were synthesised in blood cells, without specifying whether they were leucocytes or erythrocytes. Mendelsohn (1961) then showed that, following the oral ingestion of glucose in vivo or the addition of glucose to a phosphate suspension medium in vitro, human red cells were able to synthesise free and esterified fatty acids. In diabetic blood this synthesis was greatly reduced. Metabolic turnover of fatty acids in the red cells is, however, slow. Robertson & Lands (1964) showed that dietary changes in fatty acid intake take ten days to affect the fatty acid composition of the red cells. These authors suggest that synthesis of phospholipids does take place in the red cell and showed that there is an acylating enzyme system which, in the presence of ATP, converts monoacyl phospholipids to the diacyl forms (e.g. lysolecithin to lecithin). The enzyme system for the reverse process does not exist in the red cells, which suggests that there must be an exchange of phospholipids between red cells and plasma, where phospholipases do exist (Vogel & Zieve, 1962).

In the work on red cells and placental tissue involving [14-C]-glucose and fructose, the possible metabolic incorporation of sugars has been borne in mind.

Structure of the Cell Membrane

More work has been done on the structure of lipid within the membrane. One of the most extensive studies has been that of Green & Fleischer (1963) on the role of lipid in mitochondrial membranes, but the results of van Deenen (1964), Finean (1964) and others on cell membranes have shown that there are many similarities between the two.

The part of their work which is relevant to this thesis may be summarised briefly as follows. In the mammalian red cell cholesterol and phospholipid are present in the molecular ratio 1:1; cholesterol probably has a compacting action on phospholipid orientation and so has an important stabilising function. Interactions between phospholipid and protein are important, both functionally and structurally. They may interact in two ways, in both of which the bond between them is rather weak: a basic protein may form a complex with an acidic phospholipid by electrostatic interation, thus forming an ionic association; or proteins may combine with phospholipid "micelles" so that the forces involved are hydrophobic and non-ionic, thus leaving the hydrophilic groups of the phospholipid free to effect solubilisation of the lipid-protein complex. Probably both types exist in most membranes. Traditionally, lipids are water-insoluble and proteins are at least partly water-soluble, and protein therefore confers water-solubility upon the lipid. The truth is probably the reverse: a large proportion of red cell membrane protein is water-insoluble, whereas phospholipids, by forming aggregations in which the fatty acid chains are on the inside and the polar groups are on the outside, can form a dispersion of "micelles" in water which is remarkably stable.

It is not yet clear whether phospholipids can exist in the membrane in this micelle form. The membrane proteins sometimes appear to be in helical form when viewed under certain conditions and when isolated they are stable in this form. Again, it is not clear whether, in the live cell membrane, they are in a helical form or the more conventional open-chain configuration.

The conventional diagram of the arrangement of the lipid and protein of the cell membrane shows the middle or meso-layer consisting only of the fatty acid chains for the phospholipids. It is now considered likely that the mesolayer contains both protein and phospholipid in the molecular ratio 1:8.

Surface Properties of Isolated Membrane Components

The work from which many ideas of the spatial configuration of proteins and lipids had derived was studies of monomolecular layers of these substances at air-water interfaces. Proteins spread themselves on the surface of water in an extended chain configuration, whereas phospholipids orientate themselves upright with their hydrophilic groups in the water and the fatty acid chains in the air.

The factors affecting the nature of the film, and the way certain characteristics of it, in particular the surface pressure and surface potential, can be measured, have been described by Adam (1941) and more recently by Davies & Rideal (1961). Application of the theory of monolayers to phosphatides has been discussed by Anderson & Pethica (1956). Interaction between protein and phospholipid is possible in monolayers, and also adsorption by the film of other substances. A lecithin film,

for instance, will bind most cations. Such adsorption or interaction changes the characteristics of the film on compression, as measured by changes in surface pressure and potential. This thesis will describe the results obtained by compressing phospholipids in the presence of glucose, in order to see if glucose alters the configuration of phospholipids. Mixtures of phospholipid and insulin, with and without glucose, were also studied since, although no effect of insulin on the intact red cell has yet been detected, the action of insulin on the membranes of cells actively transporting glucose is almost beyond dispute.

The main part of the work on lipids described in this thesis concerns their extraction and separation from red cell membranes, but the other studies described were designed to throw some light on the problem of the carrier-glucose complex and to open up certain possibilities and lines for future work.

EXPERIMENTAL SECTION I

Phosphatase Activity in Red Cells, Kidney and Placenta.

CHAPTER 2

METHODS

General

The name "phosphatase" covers the group of enzymes which catalyse the general reaction

$$R - O - P + H_2O \longrightarrow R - O - H + HO - P .$$

Surveys of their properties and distribution have been made by Dixon & Webb (1958) and by Boyer, Lardy & Myrbäck (1960).

Two main categories are recognised: acid phosphatases with an optimal activity at about pH 5, and alkaline phosphatase with optimal activity at about pH 10. Both are rather unspecific in that they will hydrolyse most phosphoric monoesters and are widely distributed throughout the body. Each of these categories probably covers a number of slightly different enzymes.

Some more highly specific phosphatases are known, for instance ATPase which is specific to adenosine triphosphate and whose possible role in the sodium pump has already been discussed; another specific enzyme is glucose-6-phosphatase, which splits only D-glucose-6-phosphate and which is concerned with carbohydrate metabolism, probably mainly with the release of glucose from glycogen. Other specific phosphatases are pyrophosphatase and 5-nucleotidase.

The methods used for studying the non-specific phosphatases are normally based on their ability to split phosphate esters such as α-glycerophosphate or sodium phenylphosphate, which are not their normal physiological substrates (King & Wootton, 1957). The amount of phosphate liberated is determined, usually by converting it to a coloured substance whose concentration can be measured photometrically. By this means,

the relative activities of different tissues, at different pH's, have been studied. Whole tissue homogenates may be used to investigate the overall phosphatase activity of the tissue, or separate fractions, from differential centrifugation of the tissue homogenate, to investigate the localisation of the phosphatase activity within the cell particles, etc.

To localise the activity precisely, histochemical techniques are used. The method normally used is the one developed by Gomori (1952) in which phosphate liberated at the site of enzyme activity in a section of the tissue is converted to lead phosphate, then lead sulphide, which is clearly visible under the microscope.

Phosphatase activity in the haemolysed human red cell was first detected by Martland, Hansman & Robinson in 1924. It has an optimum activity at pH 5·6 - 6 and its activity in hydrolysing various substrates was found by Tsuboi & Hudson (1956) to decrease in the order phenyl phosphate, α-glycerophosphate, riboflavin-5-phosphate, β-glycerophosphate, glucose-1-phosphate. They could find no effect on glucose-6-phosphate (probably because the tissue was not fresh enough) nor on ATP (possibly because of a lack of Mg^{++}). No alkaline phosphatase has been found in red cells, although there is a high activity in the plasma.

Accordingly, in the present experiments disodium phenyl phosphate was used as the substrate for the human and guinea-pig red cell haemolysates and activity was studied in the pH range 4-8. The method followed was that of King & Armstrong as described by Abul-Fadl & King (1949), in which both phosphate and phenol are liberated. The phenol was estimated by the amino antipyrine-ferricyanide procedure of Kind & King (1954).

Materials

Guinea-pig blood was obtained from female guinea-pigs in late pregnancy. The animal was anaesthetised with 20% urethane, injected intra-peritoneally, 8 ml./kg. body weight.

One by one the foetuses were removed from the uterus by Caesarian section and released from the placental membranes. The umbilical cord was cleared so that foetal blood could be withdrawn through a hypodermic needle inserted into the umbilical artery. A total of 1-2 ml. foetal blood was thus obtained.

The foetuses were dried on the outside, weighed, and their age determined from a graph relating age to the cube root of the average weight (Huggett & Widdas, 1951).

Maternal blood was obtained by cardiac puncture after removal of the foetuses.

Syringes and vessels were heparinised.

Human blood was either obtained by venous puncture immediately before use, or was time-expired ex-blood bank. Bloods and haemolysates were stored either in the refrigerator or the deep-freeze when not being used.

Erythrocyte haemolysate. The blood was spun and the plasma and top layer of cells removed. The packed red cells were washed twice with 0·9% saline solution, each time discarding the supernatant and top layer of cells. In this way erythrocytes free from leucocytes were obtained. The washed erythrocytes were haemolysed by adding an equal volume of distilled water, then further diluted to give a predetermined concentration of haemoglobin, the standard chosen being a 1:25 dilution of human blood, haematocrit 50% and haemoglobin 100% standard. This gave a reading of 0·34 on a Hilger colorimeter using a yellow-green filter when again diluted 1:25. It was felt that this gave a more reliable standard of comparison between blood from different individuals and species than dilution by volume. It also eliminates any error involved in the colorimetric estimations due to the colour of haemoglobin.

Buffer solutions. All 0·1 Molar.

pH 2·0 - pH 6·0: mixtures of phthalate-HCl and phthalate-NaOH solutions were used. (Clarke, 1928.)

pH 6·5 - pH 7·0: veronal and HCl mixture (A).

pH 10·1: carbonate-bicarbonate mixture (B).

pH 7·5 - pH 9·5: appropriate mixtures of A and B. (Vogel, 1945.)

Each mixture was made up at least one day before using, checked on a pH meter, adjusted if necessary, and a little chloroform added before storing in the refrigerator.

Substrate. $^{M}/_{100}$ disodium phenyl phosphate.

2·18 g was dissolved in 1 litre of water, brought to the boil, cooled, and a little chloroform added.

Standard phenol. A stock solution of 1 mg./ml. was made up in 0·1 N HCl, and diluted for use with water.

Developing reagents. M/2 $NaHCO_3$ (4·2%).

0·6% 4-amino-antipyrine (A.A.P.).

A.A.P./$NaHCO_3$ mixture: equal volumes of the above solutions.

N/2 NaOH.

2·4% potassium ferricyanide, $K_3Fe(CN)_6$.

The phenol liberated from the phenyl phosphate by the action of the phosphatase reacts with A.A.P. in the presence of alkaline oxidising agents to give a substituted quinone. This has a red colour whose depth is proportional to the amount of phenol present.

Before evolving a final procedure, a few preliminary investigations were made. It was found that colour development was pH-dependent, so in order to obtain maximum development the amounts of NaOH and $NaHCO_3$ added to the system were recalculated for each pH investigated. The colour which developed was not stable: it tended to deepen in the dark and fade in the light. This was not very serious and the procedure used gave consistent results. The relation between phenol liberated and colour developed was found to be linear up to 0·06 mg. phenol/ml. in the system used; it was fairly constant from day to day, but nevertheless a new standard curve was constructed for each experiment.

Procedure.

1 ml. buffer and 1 ml. substrate were warmed in a water bath at 37°. 0·1 ml. haemolysate was added and the mixture incubated at 37° for 30 mins.

The enzymes were inactivated at the end of that time by the addition of approximately 1 ml. 0·5 n NaOH, then approximately 1 ml. 4·2% $NaHCO_3$. The exact volumes used were taken from a table devised as explained above, to bring the final pH to the optimum for the colour developing reagents.

To the mixture were added 1 ml. A.A.P./$NaHCO_3$ mixture, then 1 ml. potassium ferricyanide. Each tube was mixed by inverting it, and placed in the dark for 30 minutes. The photometric estimation was performed not before 10 minutes and not after 30 minutes after bringing back into the light.

The photometric readings were taken either on a Hilger Spekker absorptiometer, using a blue-green filter and $\frac{1}{2}$ cm. cell, or on a spectrophotometer (Optika automatic recording) set to read optical density at wavelength 508 mμ and using a 1 cm. cell. The readings of the two instruments compared well, but since in each case the activity of the enzyme was calculated from a standard curve constructed for the instrument, the results should be exactly comparable.

All readings were taken against a "blank" in which the haemolysate was replaced by distilled water, and the readings of the "Test" series were compared with those of a "Control" series in which haemolysate was present, but was inactivated with sodium hydroxide immediately and was not incubated.

The mixture for the "Standard" series was made up of 1 ml. buffer, pH 5·5, 1 ml. dilute phenol containing 0·01, 0·02, 0·03, 0·04 or 0·05 mg. phenol/ml., 0·1 ml. distilled water, 1 ml. 0·5 N NaOH and 1 ml. 4·2% $NaHCO_3$. To this was added 1 ml. each of the developing reagents.

The standard curve was constructed by plotting the readings obtained against the known phenol content.

Calculation of Phosphatase Activity.

Phosphatase activity was calculated from the standard curve: the reading obtained indicated the amount of phenol liberated from the substrate by 0·1 ml. haemolysate, that is, the phenol equivalent.

Phenol equivalent x 1000 - arbitrary Phosphatase Units, the unit being the amount of phenol which would be liberated by 100 ml. haemolysate in 30 minutes. This unit is similar to the King-Armstrong unit, which is mg. phenol liberated by a 1:10 (v/v) dilution of packed red cells in one hour for acid phosphatase and in 15 minutes for alkaline phosphatase.

EXPERIMENTAL SECTION I

Phosphatase Activity in Red Cells, Kidney and Placenta.

CHAPTER 3

RESULTS AND DISCUSSION

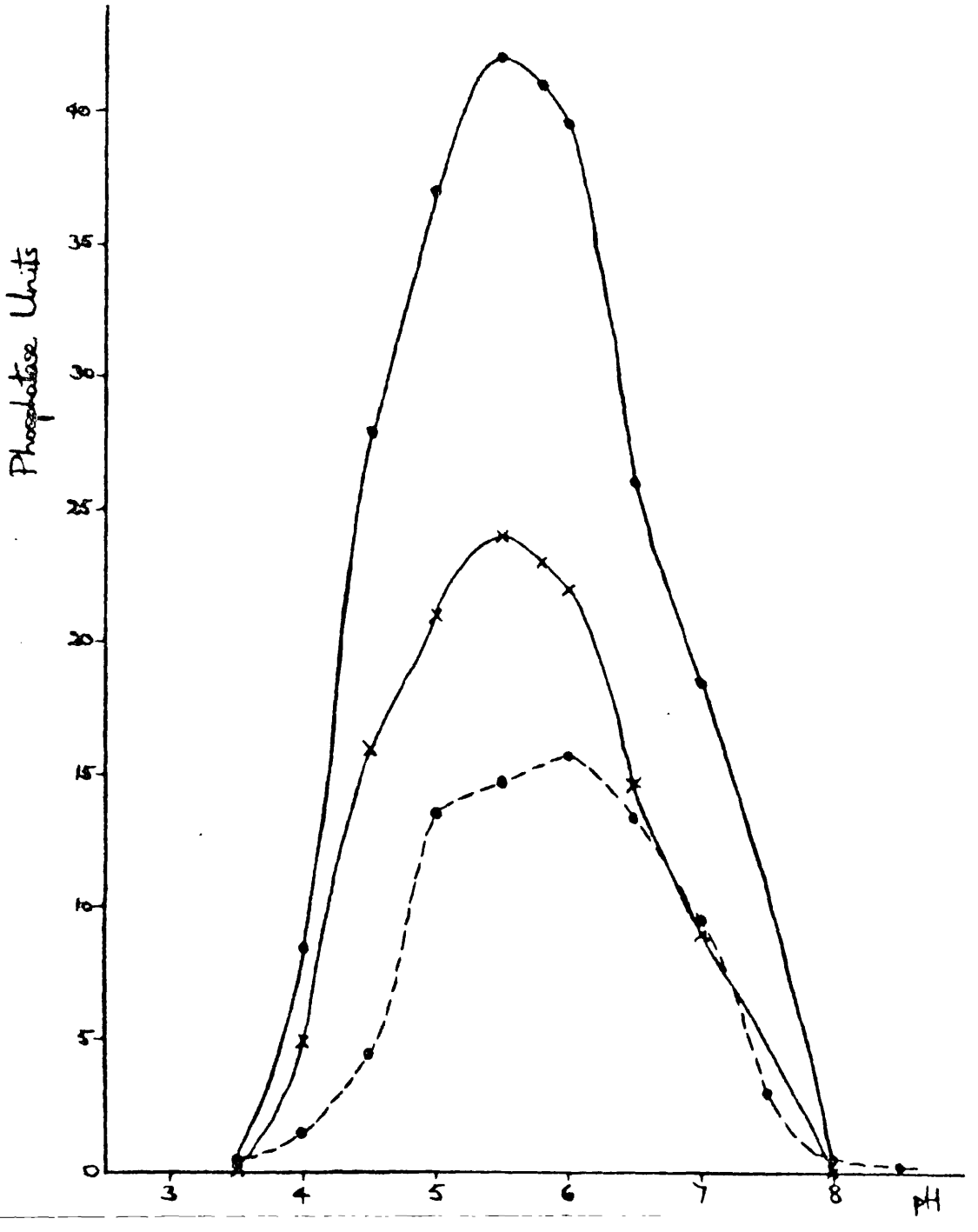


Fig. 1. pH hydrolysis curve of phenyl phosphate by haemolysed red cells.

A = human red cells ex blood bank. ●- - - -●

B = fresh maternal guina-pig red cells. ×——×

C = fresh foetal guinea-pig red cells. ●——●

Each blood sample was washed, haemolysed and diluted to give the same haemoglobin concentration.

Phosphatase Activity in Red Cells.

Comparison of Human and Maternal and Foetal Guinea-Pig Red Cell Phosphatase Activities.

Phosphatase activity was measured by the hydrolysis of phenyl phosphate over a range of pH's for human red cells and for guinea-pig maternal and foetal red cells. Typical pH curves, drawn on one graph for direct comparison, are shown in Fig. 1.

It may be seen that human red cells show optimal activity at pH 6·0 and guinea-pig, both maternal and foetal, at pH 5·5. These are near the typical pH of an acid phosphatase. Very little activity was observed above pH 8: alkaline phosphatase was absent from the red cells studied.

The activities at optimum pH were:-

Human	15·75	Phosphatase units
Maternal guinea-pig	26·2	Phosphatase units (161%)
Foetal guinea-pig	42·0	Phosphatase units (262%)

The percentages in brackets are the activities of the guinea-pig red cells relative to the human red cells.

Variations in Guinea-Pig Phosphatase Activity

Ten pregnant guinea-pigs were used altogether, and a fairly wide variation in results was obtained. Some of this was probably due to individual variance, but since red cells from the same animal gave different results on different days, it seems likely that much of it was due to deterioration of the enzymes.

TABLE I

Variations in Guinea-pig Phosphatase Activity.

Maternal Phosphatase Activity (mg. phenol liberated/100 ml. haemolysate/ 30 min.)

Refrigerator		Deep Freeze		Immediate use	
(A1)	22·5	(M5)	26·2	(M8)	27·0
(A1)	19·5	(M5)	21·0	(M9)	26·2
(M4)	24·5	(M5)	16·0	(M10)	24·5
(M4)	10·5	(M7)	26·6	(M10)	21·4
Mean	19·2	(M9)	19·5	(M11)	24·0
		Mean	21·8	(M12)	24·0
				Mean	24·5

Foetal Phosphatase Activity (mg. phenol liberated/100 ml. haemolysate/ 30 min.)

Refrigerator		Deep Freeze		Immediate use	
(F3)	32·5	(F5)	37·0	(F8)	43·0
(F3)	43·0	(F5)	32·0	(F9)	42·0
(F4)	43·0	(F5)	28·0	(F10)	38·0
(F4)	25·0	(F6)	46·0	(F10)	36·0
Mean	35·4	(F9)	34·5	(F11)	42·0
		Mean	35·5	(F12)	35·3
				Mean	39·0

Table I shows all the results obtained for guinea-pig blood, divided into categories. The "refrigerator" category refers to red cells stored for periods of more than 24 hours at 4^{o}, "deep-freeze" to those which spent at least 24 hours at -10^{o}. In the "immediate" category, the activity was estimated on the day of withdrawal from the animal and the haemolysate was kept on ice during the day. Two results with the same code number refer to experiments on two different days using the same haemolysate, except M10 and F10 where two estimations were carried out on the same day.

It may be seen how quickly the haemolysate lost its phosphatase activity. The experiments using M5 and F5, for instance, were carried out on the second, sixth and ninth days after withdrawal of the blood from the animal. The rate of glucose transfer in human red cells, on the other hand, is unaffected by prolonged storage of the blood at 4^{o}, and separated red cells may be stored for a few days without serious deterioration.

Variation with Foetal Age

The phosphatase activity of the foetal red cell appeared to depend partly on its age. The guinea-pigs used in these experiments were all in late pregnancy and the results obtained were for only a narrow range of ages.

	Foetal Age	Phosphatase Activity
F10	46 days	38 P.U.
F8	50 days	43 P.U.
F11	55 days	42 P.U.
F9	59 days	42 P.U.
F5	60 days	37 P.U.
F12	63 days	35 P.U.

Effect of Inhibitors on Phosphatase Activity

Little work has been done to find substances which inhibit acid phosphatase activity. Boyer, Lardy & Myrbäck (1960) state that red cell phosphatase may be distinguished from prostatic acid phosphatase by the fact that it is not inhibited by tartrate, but that it is by copper ions and slightly by fluoride and cyanide. It is known (LeFevre, 1948) that copper ions have no effect on glucose transfer in the red cell. Mio, Hashimoto & Yoshikawa (1964) found purified horse red cell acid phosphatase to be inhibited completely by heavy metals, but not by N-ethyl maleimide at 1 mM. They found that p-chloromercuribenzoate had a marked inhibitory action, however, and calculated that if the enzyme had a molecular weight of 30,000 - 60,000 and that if the inhibitor acted by attacking sulphydryl groups, then each molecule of enzyme had 1 - 2 sulphydryl groups closely related to its phosphatase activity.

Glucose-6-phosphatase (which would probably not be active under the conditions of these experiments) has been studied more extensively and is inhibited by phosphate, glucose, fluoride, copper ions, phlorrhizin and N-ethyl maleimide (Ashmore & Webber, 1959). ATPase from rabbit red cells is inhibited by phloretin, phlorrhizin and glucose at pH 7·4 (Laris, Novinger & Calaprice, 1960).

Little appears to be known about the effect on red cell acid phosphatase of phloretin (which inhibits glucose transfer in red cells), phlorrhizin (which inhibits glucose transfer in red cells and glucose reabsorption in kidney tubules), or N-ethyl maleimide (a sulphydryl reagent known to inhibit glucose transfer non-competitively).

For the investigation of the effect of inhibitors on phosphatase activity, the incubation mixtures were made up as before, but 0·1 ml. inhibitor solution was added before the addition of haemolysate or homogenate.

The inhibitor solutions were made up as follows:-

Phloretin. Stock solution containing 1% phloretin in absolute ethanol was diluted with 0·9% NaCl, usually to give 1:5,000. In an incubating volume of 2·3 ml. this gave a concentration of 2×10^{-3} mM.

Phlorrhizin. 9·44 mg. was made up to 50 ml. with 0·9% NaCl. In an incubating volume of 2·3 ml. this gave a concentration of 2×10^{-2} mM.

Glucose. D-Glucose was made up with distilled water to give incubation concentrations varying between 10 mM and 10^{-2} mM. These were made up freshly for each experiment because glucose tends to undergo configurational changes in solution.

NEM (N-ethyl maleimide). A solution 25 mg./ml. was made up in 10% alcohol. NEM acts as an irreversible inhibitor of glucose transfer, unlike the others which act competitively, so probably acts as an enzyme inhibitor by forming an irreversible complex. In view of this, two ways of using it were tried:-

(1) Intact red cells were incubated with 8 mM NEM for 30 min. at 20°, then the cells were washed, haemolysed and incubated with substrate.

(2) Haemolysed red cells were incubated with the NEM at pH 7·5, then substrate and more buffer were added, at the higher temperature, for a further 30 minutes incubation.

TABLE II

Inhibition of Guinea-Pig Red Cell Phosphatases by Phloretin, Phlorrhizin and Glucose.

Inhibitor	Conc. (mM.)	No. of Expts.	Phosphatase Activity		Inhibition	
			Non-treated	Treated	Observed	Calculated
MATERNAL						
Phloretin	3×10^{-5}	3	24·0–26·2	23·5–27·5	0·4%	2%
Phloretin	2×10^{-3}	5	24·0–27·0	23·0–25·0	6·25%	55%
Phlorrhizin	5×10^{-2}	4	19·5–26·2	20·0–26·8	3·3%	25%
Phlorrhizin	4×10^{-1}	1	24·5	26·5	-8·0%	75%
Glucose	5	3	26·2–27·0	5·0–26·0	47·5%	
FOETAL						
Phloretin	3×10^{-5}	3	38·0–42·0	37·5–44·4	-0·25%	2%
Phloretin	2×10^{-3}	5	35·3–46·0	36·0–41·5	5·9%	55%
Phlorrhizin	5×10^{-2}	4	34·5–42·0	33·0–43·6	3·2%	25%
Phlorrhizin	4×10^{-1}	1	38·0	38·5	-1·3%	75%
Glucose	5	3	42·0–46·0	15·8–25·8	53·5%	

Results

The effects of phloretin, phlorrhizin and glucose on the acid phosphatase activity of the guinea-pig red cells at pH 5·5 are shown in Table II. In each experiment "non-treated" and "treated" were estimated on blood from the same animal, at the same time, and the percentage inhibition determined for that animal. "Observed Inhibition" is the mean of the results obtained. "Calculated Inhibition" is the percentage inhibition which would have been expected if acid phosphatase were concerned directly with glucose transfer, and was calculated from the values obtained by Sen & Widdas (1962b) for the half-saturation constants of phloretin and phlorrhizin using human erythrocytes at pH 7·4. These constants are similar to those also obtained for foetal guinea-pig red cells (Dawson, unpublished observation). The inhibitory effects of phloretin and phlorrhizin were studied between pH 4 and 7, and were found to be normally greatest between pH 5 and 6. At no pH did the inhibition by phloretin approach that calculated from its inhibition of glucose transfer. The instability of the red cell membrane at pH 5·5 made it impossible to obtain a reliable value for inhibition of glucose transfer by phloretin. However, measurements by LeFevre and Marshall (1959) of the uptake by red cells of phloretin show that the cell:medium ratio is much greater at pH 5·5 than at pH 7·4. Since they also found a definite correlation between uptake and inhibition, it would be expected that the inhibition by phloretin of glucose transfer, if measurable, would be greater at the lower pH.

The same table shows that phlorrhizin, in a concentration where it would be expected to give about 75% inhibition, actually gave a small potentiation to the phosphatase activity. Although this was only a single experiment with the maternal and foetal red cell phosphatase preparations, there was good agreement between them. This result further emphasizes the lack of correlation with the expected inhibition of glucose transfer.

Glucose was found to inhibit maternal and foetal red cell phosphatases at all concentrations between 0·5 and 10 mM; the extent was variable but the maximum inhibitory effect was usually found at 5 mM. This is equivalent to a blood glucose level of 90 mg./100 ml. blood and is similar to the half-saturation constant of glucose in red cell transfer. Higher concentrations showed no progressive inhibition, as would be expected, nor was it possible to demonstrate a clear competitive effect between the inhibitory action of glucose and phloretin or glucose and phlorrhizin. The apparent coincidence of the effective concentration of glucose and the half-saturation constant for glucose transfer was not further studied.

Inhibition by N-ethyl Maleimide

Two experiments were performed. In the first, intact human red cells were pre-incubated with 8 mM NEM for 30 minutes at 20°. The red cells were then washed, haemolysed, and phosphatase activity estimated at pH 5·8. The results are shown in Table IIIA.

TABLE III

Inhibition of Red Cell Phosphatase Activity by N-Ethyl Maleimide.

A. Preincubation of Intact Human red cells with 8 mM NEM for 30 minutes at 20°, at different pH's.

Preincubation pH	Phosphatase Activity at pH 5·8	% Inhibition
Non-treated	13·5 P.U.	
5·5	0·7	94%
6·5	0	100
7·5	0	100

B. Preincubation of Haemolysed Guinea-pig red cells with 0·5 mM NEM for 30 minutes at 20°, at pH 7·5. Also with 5 mM glucose.

	MATERNAL		FOETAL	
	Phosphatase Activity at pH 5·8	% Inhibition	Phosphatase Activity at pH 5·8	% Inhibition
Non-treated	24·0 P.U.		39·0 P.U.	
NEM 0·5 mM	7·0	71%	12·5	68%
Glucose 5 mM	8·3	65%	10·0	74%
NEM + Glucose	7·5	69%	0	100%

It may be seen that there was higher inhibition at the higher pH's. This agrees with the results obtained by Dawson & Widdas (1963) for inhibition of glucose transfer. The results do not agree, however, in the degree of inhibition obtained, since these workers found it impossible to obtain more than 97% inhibition using concentrations of even 18 mM NEM. They found, moreover, that incubation with 8 mM NEM at 20°, pH 7·4, for 30 minutes produced under 70% inhibition of glucose transfer.

In the second experiment, haemolysed guinea-pig red cells were pre-incubated with 0·5 mM NEM for 30 minutes at 20°, pH 7·5, then 0·2 ml. aliquots were added to tubes containing buffer and substrate, and phosphatase activity over the pH range 4–7 was estimated. The maximum inhibitory effect was at pH 5·8, and the results shown in Table IIIB were calculated for that pH.

It may be seen that percentage inhibition at this concentration was lower than in the previous experiment, but was still much higher than the inhibition of glucose transfer found by Dawson & Widdas (1963). The disintegration of the cell membranes and exposure of intracellular proteins may have had an effect in reducing the inhibitory effect of NEM, but a mopping up of NEM by exposed groups would have been expected to lower the inhibition still further.

The response of the foetal phosphatase activity to NEM in the presence of glucose was total inhibition at all pH's except pH 5·0. In the maternal red cell, the effect of NEM + glucose was intermediate between the two separate effects.

Histochemical Investigation of Phosphatase Activity in Guinea-Pig Placenta and Kidney.

Preliminary estimations of the acid and alkaline phosphatase activity in guinea-pig placenta and in maternal and foetal kidney showed that activity in both pH ranges was considerable. The placenta is an area of large-scale movement of materials across membranes, and an area where a facilitated diffusion of glucose has been postulated (Widdas, 1951). No reference could be found in the literature to histochemical localisation of phosphatases within the guinea-pig placenta. Similarly, no reference could be found to the localisation of phosphatases in the foetal guinea-pig kidney.

If kidney phosphatases were involved in glucose transfer, then their distribution in the foetal kidney would presumably depend on the degree of development of the glucose reabsorption mechanism. In order to discover whether the foetal guinea-pig kidney in late pregnancy (60 days) was capable of actively reabsorbing glucose, urine was withdrawn from the bladder with a hypodermic syringe and tested for the presence of reducing sugars. The test was negative, which suggests that the reabsorption mechanism was already present.

The histochemical techniques were carried out by the late Dr. Audrey M. Bond, but not interpreted.

The kidneys, maternal and foetal, and the placenta were removed from the guinea-pig immediately after withdrawal of the blood for red cell phosphatase estimations. The materials were fixed in acetone, double-embedded in wax and cut into 5μ sections.

The revised method of Gomori (1946) was used: the sections were incubated in a mixture of glycerophosphate and lead nitrate at either pH 5·0 or 10, for a known length of time, then treated with ammonium sulphide. In this way lead sulphide was deposited at the sites of phosphatase activity and was visible under the microscope either as black particles or as a brown stain.

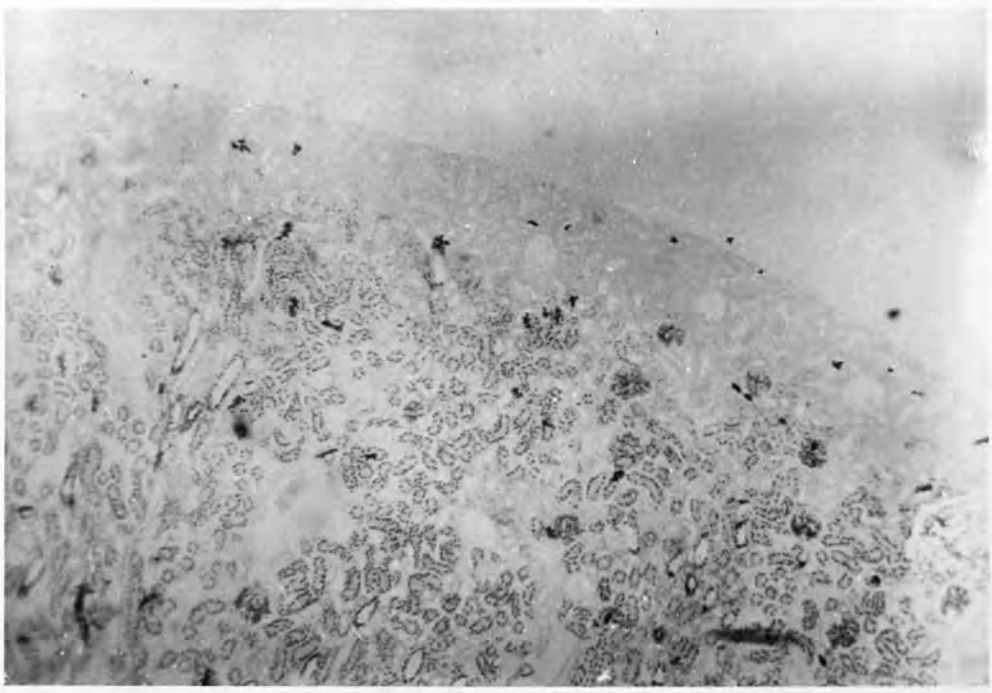
The relative activities of the phosphatases in the tissues was gauged by the incubation time required to give a clear histochemical effect. The following incubation periods were used to give the best results:-

Acid phosphatase	Maternal kidney	60 minutes
pH 5·0	Foetal kidney	60 minutes
	Placenta	5 hours
Alkaline phosphatase	Maternal kidney	30 minutes
pH 10	Foetal kidney	60 minutes
	Placenta	10 minutes

Fig. 3 shows maternal and foetal kidney sections incubated at pH 5·0; fig. 4 shows them at pH 10·0. Fig. 5 shows guinea-pig placenta sections at pH 5·0 and pH 10·0.

The distribution of activity in the kidney was similar in maternal and foetal sections and agreed with published results. Gomori (1946), for instance, described alkaline phosphatase activity as being confined to the brush borders of the proximal convoluted

(a)



(b)

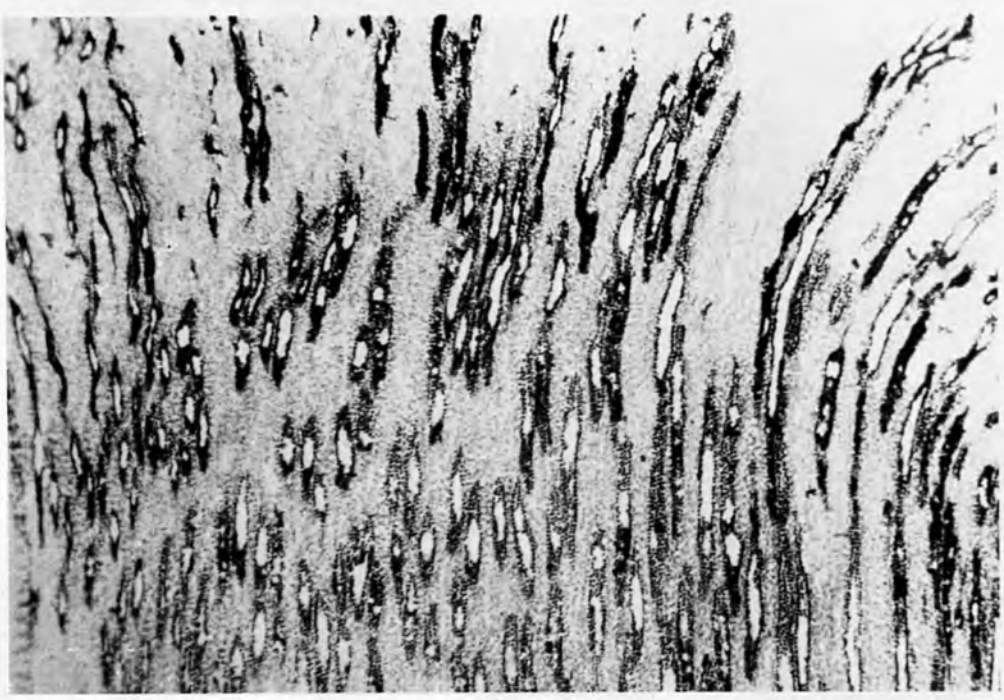


Fig. 3A. Acid phosphatase activity in maternal guinea-pig kidney, at pH 5·0. Magnification x 30. (a) Cortex; (b) medulla.

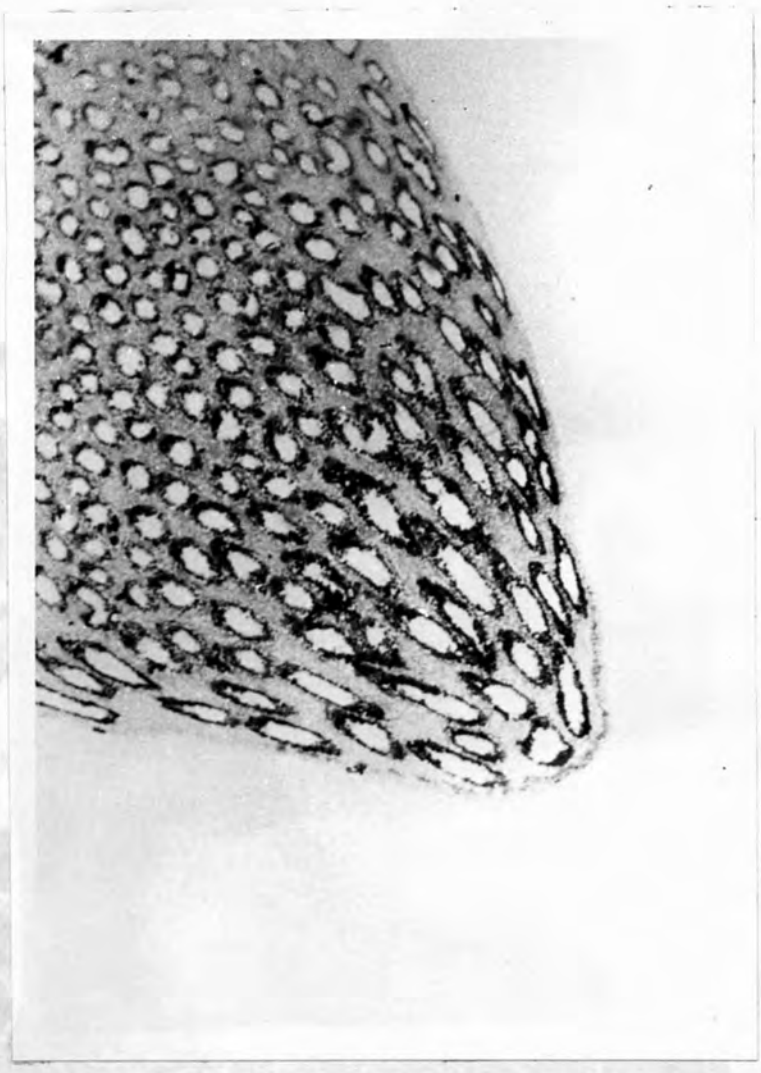
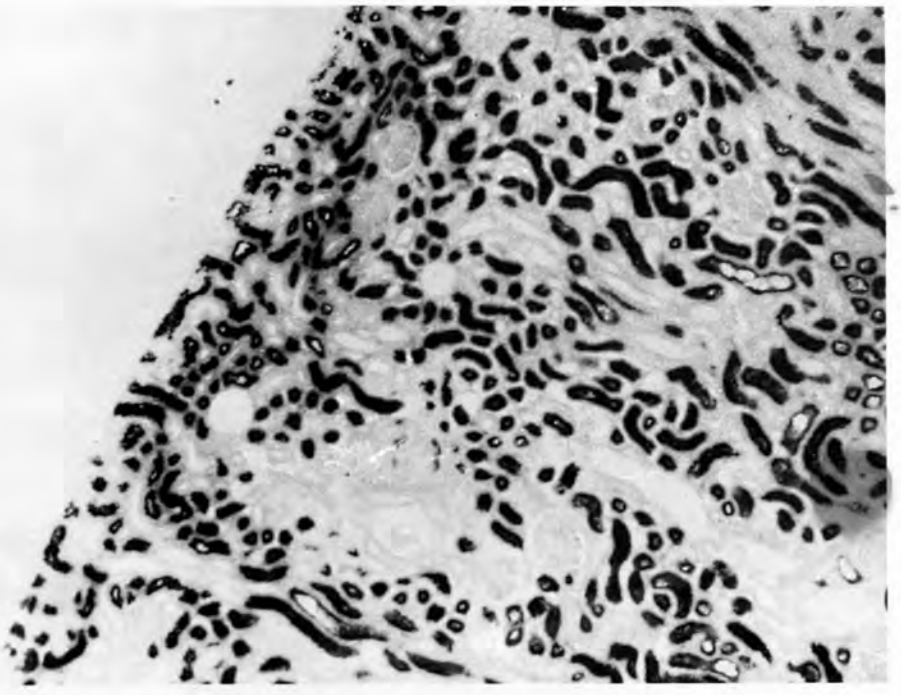
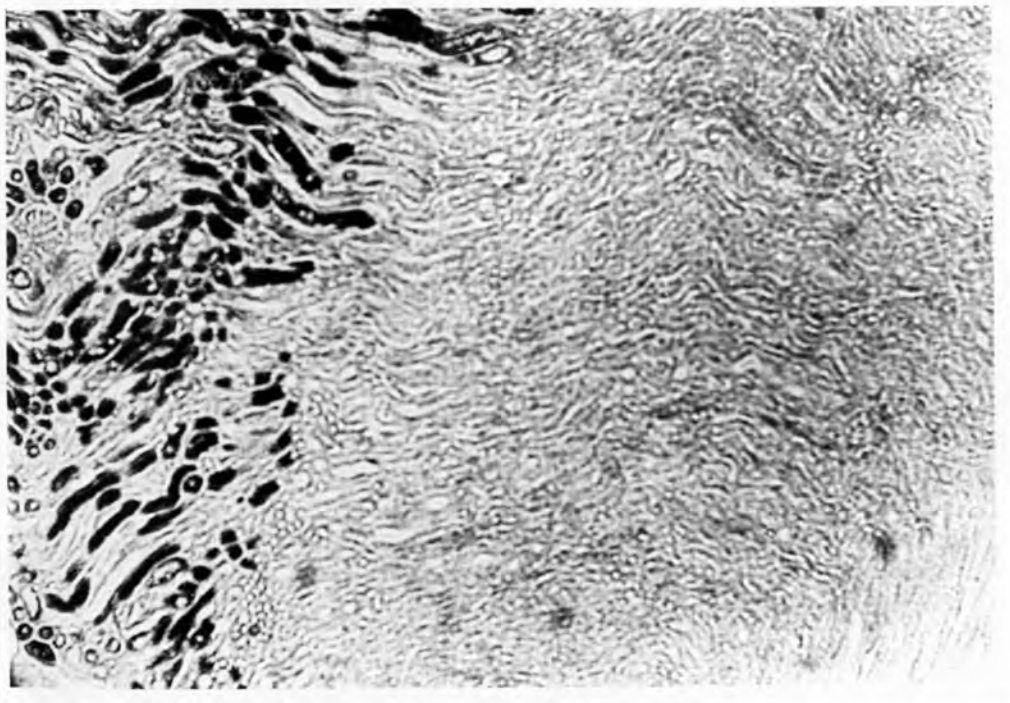


Fig. 3B. Acid phosphatase activity in foetal guinea-pig kidney at pH 5.0, showing the medullary region. Staining in the cortical region was too faint for effective photography. Magnification x 30.



(a)



(b)

Fig. 4A. Alkaline phosphatase activity in maternal guinea-pig kidney at pH 10.0. Magnification x 30. (a) Cortex; (b) medulla.

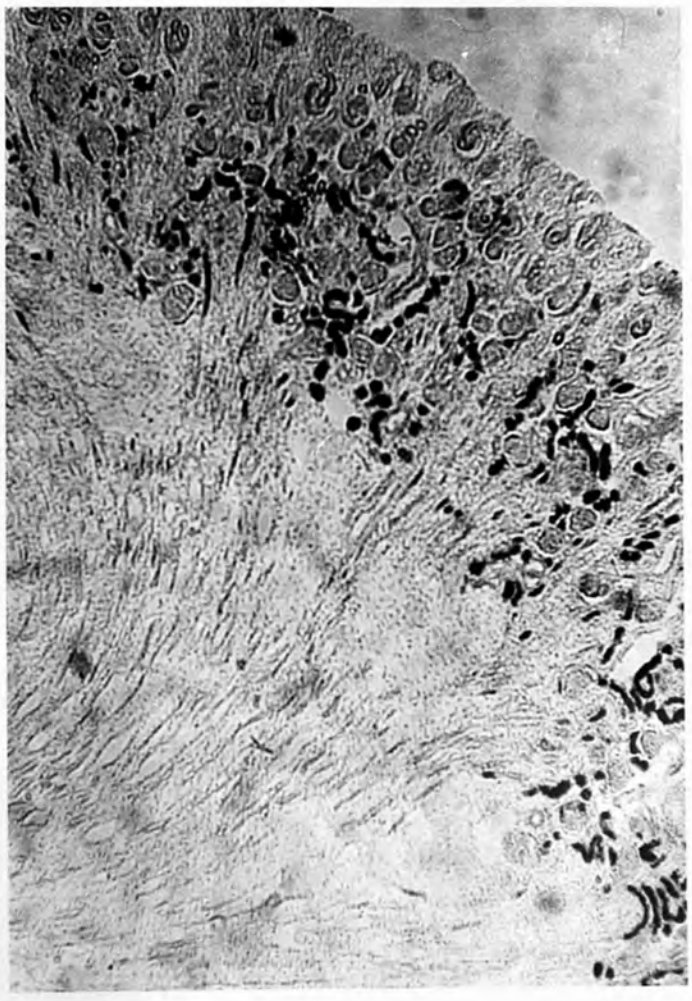
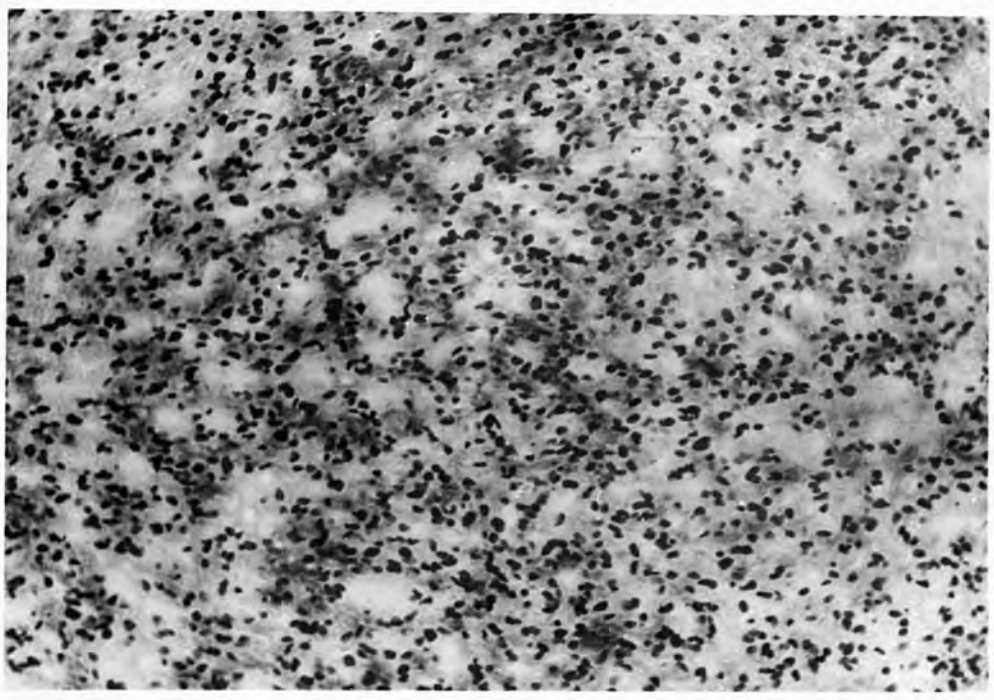


Fig. 4B. Alkaline phosphatase activity in foetal guinea-pig kidney at pH 10·0, showing medulla and cortex. Magnification x 30.

(a)



(b)

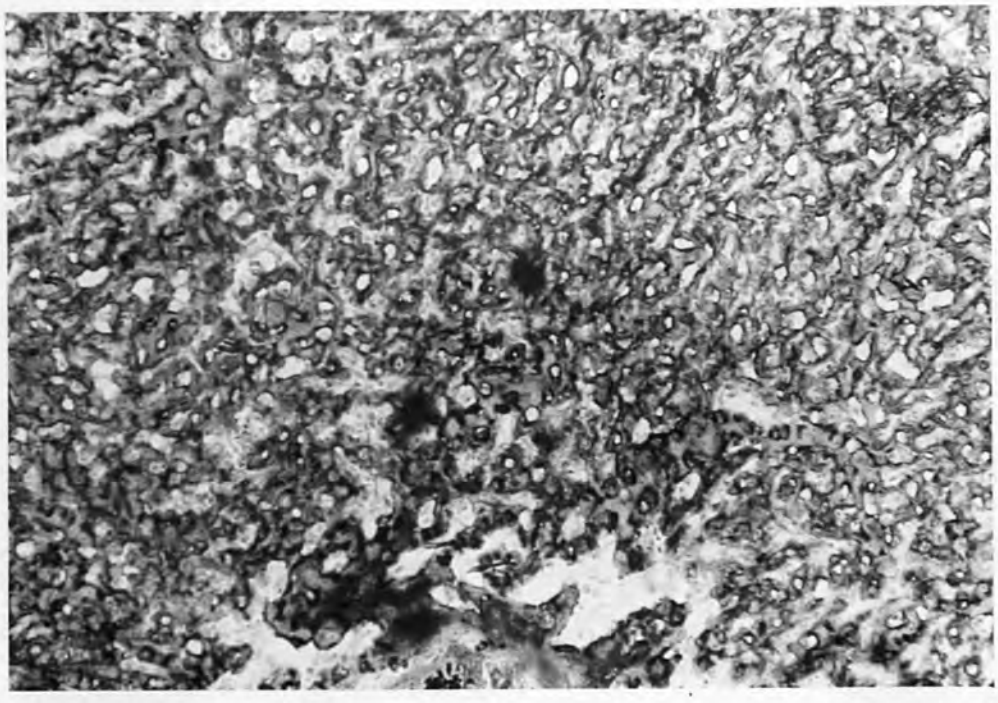


Fig. 5. (a) Acid and (b) alkaline phosphatase activity in guinea-pig placenta, at pH 5·0 and pH 10.0 respectively. Magnification x 100.

tubule in all the species studied. Acid phosphatase, on the other hand, he found to be variable in distribution, sometimes only visible in the glomerulus and sometimes visible in both cortex and medulla.

Dr. Bond's findings of acid phosphatase distribution in the guinea-pig kidney were fairly consistent: it was found in all parts of the nephron, including the loop of Henle, and in both the maternal and foetal sections was most marked in the collecting ducts. In most parts the area round the nucleus was stained, but in the collecting ducts and the limbs the staining was throughout the cell.

Fig. 5 shows that the alkaline phosphatase activity of the guinea-pig placenta, which is remarkably high, is generalised throughout the cells of the tissue. The acid phosphatase, which has a low activity, stains mostly in the region of the cell nuclei. These findings are in agreement with those of Ahmed & King (1959) for human placenta. These authors also used differential centrifugation of placental homogenates; they found acid phosphatase to be present mainly in the cytoplasm and alkaline phosphatase mainly in the microsomes and cytoplasm.

A few experiments were carried out in which inhibitors were added to the incubation mixtures. 8 mM NEM completely inhibited alkaline phosphatase in both maternal and foetal kidney sections. Foetal alkaline phosphatase was noticeably inhibited by phloretin, phlorrhizin and ouabain; maternal was unaffected. The acid phosphatase of both maternal and foetal sections was strongly inhibited by phloretin in the medulla, and to a much lesser extent in the proximal tubules. Phlorrhizin and ouabain had no effect at pH 5·0, but phlorrhizin appeared to give some inhibition at pH 4·0.

Placental alkaline phosphatase was strongly inhibited by NEM. Acid phosphatase was possibly inhibited by phloretin when they were incubated together over long periods.

DISCUSSION

The distribution of phosphatases in the foetal and adult guinea-pig is at first sight suggestive of a role in glucose transfer. The foetal red cell, which has a high rate of glucose transfer, has a greater acid phosphatase activity than the adult red cell, in which the rate of glucose transfer is low. The kidney in both the late foetal and the adult guinea-pig (both of which have a glucose reabsorption mechanism) show acid phosphatase activity. N-ethyl maleimide, a known inhibitor of glucose transfer in the red cell, inhibits acid phosphatase activity in both the red cell and the kidney. Glucose inhibits acid phosphatase activity in the red cell at 5 mM; 5 mM is also the half-saturation constant of glucose for the transfer mechanism (Sen & Widdas, 1962a).

On closer investigation, however, discrepancies appear. The acid phosphatase level in the human red cell is lower than that in the adult guinea-pig, in which the rate of glucose transfer is low. The red cell acid phosphatases deteriorate rapidly, even when stored at 4^{o}, while the power of the red cell to transfer glucose remains at the same level for many weeks if stored at 4^{o}. This cuts out the rapidly-deteriorating enzymes such as glucose-6-phosphatase as being involved in glucose transfer in the red cell, but it is still conceivable that there are other slowly-deteriorating enzymes, whose activity is measured

by the method used, which could be involved. To elucidate this it would be necessary to separate, by electrophoresis for example, all the enzymes contributing to the hydrolysis of disodium phenyl phosphate and to measure the rate of deterioration for each one.

Inhibitor studies with phloretin and phlorrhizin do not show any correlation between glucose transfer and acid phosphatase inhibition in the red cell. If the enzymes had been involved in glucose transfer higher rates of inhibition might have been expected in the foetal than in the adult red cell, whereas in fact the foetal enzymes were slightly less inhibited. Both in the maternal and in the foetal red cells the rates of inhibition were only a fraction of those predicted from the half-saturation constants of the inhibitors for the transfer mechanism.

The effect of glucose on red cell acid phosphatase activity may be explained in several ways which do not involve glucose transfer across the membrane. The most likely of these is an involvement of the acid phosphatase in the glycolytic processes of the red cell.

N-ethyl maleimide is not a specific inhibitor of glucose transfer, but is a "protein reagent" which reacts with groups such as the sulphydryl and amino groups irreversibly. The fact that it does inhibit glucose transfer suggests that a protein, or lipo-protein, or at any rate an amino-group, is involved in the transfer mechanism.

The distribution of acid phosphatase in the guinea-pig kidney does not correlate with the sites of glucose transfer. In the maternal kidney its distribution is generalised, whereas glucose reabsorption takes place only in the proximal convoluted tubule (Walker et al, 1941),

and in the foetal kidney acid phosphatase is found mainly in the medulla. Moreover, in the proximal tubules, the enzyme is active throughout the cytoplasm, whereas if it were involved in transport mechanisms it would be expected on the luminal surface of the cell.

There is a better correlation between alkaline phosphatase distribution and sites of glucose transfer. Alvarado and Crane (1962) have suggested that there are two components to glucose reabsorption: (1) an entry component which is independent of energy supplies and which probably involves a membrane carrier; (2) an accumulation component which is dependent on energy supplies. The entry component, in this scheme, is analagous to the transfer mechanism in red cells. Since alkaline phosphatase is absent from red cells, the enzyme, if involved at all in glucose transfer, must act during the second "active" stage.

It was concluded, as a result of this work, that there was no satisfactory correlation between phosphatases and facilitated glucose transfer.

Subsequent work was directed towards a study of membrane lipids and to seek evidence of a complexing reaction with glucose. This work and the techniques used in it form the second part of this thesis.

EXPERIMENTAL SECTION II

Phospholipids from Cell Membranes

CHAPTER 4

Methods of Extraction and Separation of Phospholipids

PART 1 - EXTRACTION OF LIPIDS.

In the Introduction some mention was made of the different types of lipids to be found in cell membranes, of their association with protein and of their ability to solubilize non-lipid substances.

Their very nature makes the lipids, and particularly the phospholipids, difficult to obtain in the pure state, and only within the last ten years have some of the problems involved been solved. Reviews of the subject have been written by Lovern (1955) and Marinetti (1962).

It has been found that triglycerides and sterols are easily extractable from most tissues by most fat solvents, for instance petroleum spirit, but that to extract phospholipids an alcohol is necessary to liberate them from their protein-bound form. Ethanol and methanol, however, are not good solvents, and cephalins are insoluble in either. Acetone similarly is good for freeing the phospholipids but is a poor solvent. To overcome these difficulties, combinations of solvents are often used, chloroform-methanol being a particularly common choice, or several solvents are used in succession.

Having extracted the lipids from the tissue as completely as possible, the next problem is that of non-lipid contaminants, which arise from two sources. First, many solvents, particularly acetone and alcohol, readily extract urea, amino-acids, nitrogenous bases, sugars, etc. from wet tissue; second, substances normally insoluble in fat solvents are readily soluble in the presence of phospholipids. The extraction of contaminants may be largely overcome by drying the tissue before extraction, or by re-extraction of the alcoholic extract with petroleum spirit. The contamination due to solubility in phospho-

lipid may be overcome by washing the extract with water, for instance by the procedure of Folch, Ascoli, Lees, Meeth & LeBaron (1951), but some lipid is invariably lost at the same time. A non-aqueous method is the chromatographic technique described by Biezenski (1962), where the contaminated lipid is run down silicic acid-impregnated filter paper with chloroform-methanol 4:1 - the contaminants remain adsorbed to the silicic acid but the lipid is completely eluted.

A method for the virtually complete purification of phospholipids was described by Hanahan, Dittmer & Warashina (1957), which involved re-extraction from ethanol-ether into petroleum spirit, then precipitation of the phospholipids in cold acetone.

Several of these methods were tried in the present experiments and will be described in this chapter. The ultimate aim of this work was to extract from red cell membranes and from placental tissue the complex between glucose and the hypothetical membrane carrier, and this put particular requirements on the method of extraction to be used. For instance, a washing procedure which might cause the glucose complex to dissociate would be undesirable. Also, a very lengthy procedure was considered unwise in view of the fact that phospholipids undergo chemical changes during treatment, for instance plasmalogens tend to lose their aldehyde moiety and become lyso-compounds, and the double bonds of the fatty acids become oxidised (Wilke, Eberhard & Schulz, 1959). Moreover, the method of separation to be employed - chromatography on a silicic acid column - would at the same time purify the phospholipids to some extent. So the methods chosen were as far as possible quick, and what was lost in purity was gained in the quantity of lipid recovered.

Materials

Human Blood was either fresh venous blood taken in small quantities and used immediately, or was time-expired, ex-blood bank, stored at 4^{o} and used within a few weeks of the expiry date.

Sheep Placental Tissue: Cotyledons from the placentae of Welsh mountain sheep were obtained in the course of other experiments at the Department of Physiology, St. Mary's Hospital Medical School, London. After separation of the foetus, the placenta was removed, the cotyledons dissected off rapidly, kept on ice and used within the hour.

Sheep Blood was obtained at the end of the procedure described above, by withdrawal from an artery.

Preparation of Red Cells.

The blood was centrifuged and the plasma and top layer of cells removed. The red cells were washed three times with phosphate-buffered saline, pH 7·4. If the supernatant was more than a pale pink, haemolysis was judged to be too great and the blood was discarded.

Preparation of Red Cell Ghosts.

The red cells were washed as before, but in the refrigerated centrifuge at 5^{o}. Haemolysis was achieved by the addition of 12 ml. distilled water to 3 ml. packed red cells, giving a medium containing 0·2% NaCl. The haemolysate was spun at 11,000 r.p.m. for 15 minutes at 5^{o}, and pale pink ghosts were obtained.

Preparation of Placental Tissue.

The cotyledons were freed of connective tissue, washed in 0·9% NaCl to remove blood from the outside, weighed, then homogenised in a Waring blender with the chosen extraction solvent.

Preliminary Investigations into Methods.

1. Extraction of Ghosts with Chloroform-Methanol $2\frac{1}{2}$:1.

10 ml. human erythrocyte ghosts were placed in a narrow glass tube with a plug of glass wool in its pointed tip. 100 ml. chloroform-methanol $2\frac{1}{2}$:1 was run through. The method was unsatisfactory because the solvent ran through too quickly, but the extract obtained was evaporated to dryness under reduced pressure in a stream of nitrogen at 37^{o}. The dried extract was redissolved in 2 ml. chloroform-methanol $2\frac{1}{2}$:1 and its surface properties were investigated by the methods to be described.

2. Extraction of Ghosts by a Series of Solvents.

10 ml. human erythrocyte ghosts were mixed with twice their volume of Hyflo-Supercel (Hopkins & Williams), packed loosely into a column with a glass wool plug in its drawn-out tip, then the following solvent mixtures were run through from a burette in succession:

3% ether in petroleum spirit,

40% ether in petroleum spirit,

20% ethanol in ether.

This method was also rather unsatisfactory because the column became packed too tightly so that the solvent flow was very slow. Each extract was evaporated to dryness at reduced pressure in a stream of nitrogen at 37^{o}, and each was investigated for its ultra-violet spectrum and its surface properties.

Ultra-violet Spectroscopy.

Each lipid and lipid solvent has a spectrum in the ultra-violet light region which is fairly characteristic of it. It was thought that to record the spectrum of a solution of an extract would be a convenient way of gaining some knowledge of its constituents.

A double-beam automatically-recording spectrophotometer (Optica) was used, switched to record optical density. The usual range of wavelength covered was 200-400 mμ. and each extract was run against a reference cell containing pure solvent.

A suspension of human red cell ghosts, run against water, gave peaks of optical density at 230, 265 and 275 mμ., and also at 400 mμ., but this was probably due to derivatives of haemoglobin. Most of the extracts gave some of the peaks seen with the ghost suspension. Peaks at 255, 261 and 269 mμ. were probably due to contamination with benzene.

Using extraction method 2, the following peaks were observed:-

3% ether in petroleum spirit:	225, 235 and 260 mμ.
40% ether in petroleum spirit:	225, 235 and 255 mμ.
20% ethanol in ether:	230-245 and 270 mμ.

From these results it was concluded that alcohol was necessary at some stage to extract the red cell ghosts completely.

Recording Surface Behaviour on the Langmuir Trough.

It has already been mentioned in the Introduction that constituents of cell membranes such as proteins and lipids form a monomolecular film when applied at an air-water interface, which shows characteristic properties when compressed. The properties are dependent on the number

of molecules present, and on their behaviour in respect to each other and to solute molecules in the liquid phase (or substrate) (Adam, 1941; Davies & Rideal, 1961).

A distinction is made between "gaseous" films in which each molecule is separate and independent of the others, and "liquid" films in which the molecules tend to coalesce. In practice, as the surface area available to the molecules is reduced, there is a rise in surface pressure due to the movements of the molecules which is analogous to gaseous pressure. At some point the molecules may start to coalesce, and at a later point a state of maximum surface pressure is reached when the molecules can no longer be packed more tightly together. If the area is reduced further at this point, the film either crumples, collapses or forms a bimolecular layer. The collapsing pressure is usually constant for a particular substance so long as the conditions, and particularly the temperature, are kept constant. Another characteristic is the area which each molecule occupies at this pressure, which is known as the molecular area.

The ionised groups on both protein and phospholipid tend to shift in their relative positions when the film is compressed, giving rise to changes in surface potential which may be measured in various ways. The behaviour of the film is also dependent on the nature of the substrate, that is the presence or absence of solutes in the aqueous liquid phase. Lecithin, for instance, exerts only a low surface pressure over a substrate of pH 10 compared with its pressure at pH 7. The surface potential also falls above pH 7, because the zwitterion structure of lecithin is affected by pH (Anderson & Pethica, 1956).

The methods which may be used for studying these two main characteristics of monolayers, surface pressure and surface potential, have been discussed by Rothen (1956), and the ones employed in these experiments are as described by him.

Measurement of Surface Pressure.

A Langmuir-Adam trough was used, made of glass, with the sides ground flat and covered with a thin, smooth layer of paraffin wax. The length of the trough was 30 cm., the width 12.5 cm. It was filled with 0.9% NaCl so that a slight convex meniscus was formed, then the surface was cleaned meticulously by pushing across it barriers of waxed glass. To keep the surface free of dust, the whole apparatus was enclosed in a perspex box and the lid was closed as soon as the surface had been cleaned.

To measure surface pressure, the vertical pull method was used. A thin glass cover slip was thoroughly cleaned by washing in chromic acid, running water, then freshly distilled water, and was suspended from the lever of a torsion balance so that it dipped into the saline and was held there by the surface tension of the liquid. Surface pressure is equal to a decrease in surface tension: when the monolayer was compressed, surface pressure increased and the glass slide was pushed out of the water. Movements of the lever caused alterations in the amount of light falling on a photocell, the changes in potential from which were indicated on a pen-writing meter.

The apparatus was calibrated in order to convert millivolts back into surface pressure: a 200 mg. weight was suspended from the lever in place of the glass plate and caused a deflection on the scale of 8.2

divisions. 200 mg. corresponds to a force of $981 \times \frac{200}{1000} = 196$ dynes, but the perimeter of the glass slide was 4 cm., so that a reading of 8·2 divisions would correspond to a surface pressure of $\frac{196}{4} = 49$ dynes/cm. Since a surface pressure of 49 dynes/cm. causes a deflection of 8·2 divisions, one division corresponds to a surface pressure of $\frac{49}{8{\cdot}2} = 6$ dynes/cm.

When the surface had been cleaned and the glass slide put in position, the substance to be studied was applied to the surface from a syringe whose plunger was controlled by a micrometer screw gauge (Agla micrometer syringe). A rotation of the screw through 360 degrees passed 50 divisions and expelled 10 $\mu\ell$. Thus a known volume of solution of known concentration of the substance to be studied could be applied in small droplets to the surface of the saline. The solvent chosen for the solution was, if possible, one which evaporated rapidly, leaving the substance in a dispersed state at the air-water interface. It was found necessary to leave phospholipids a minute or two to disperse on the surface, whereas proteins needed several minutes.

A waxed barrier at 24 cm. from the end of the trough was then mechanically pushed slowly along the trough towards the glass slide. If the right amount of material had been applied to the surface, the surface pressure began to rise when the barrier was about half-way along, that is at about 12 cm. The pressure continued to rise as the surface area available to the molecules decreased, then reached a maximum value which was maintained if the film was stable. The barrier stopped short of the glass slide, at about 3 cm.

If this process was repeated several times with the same material, carefully cleaning the surface between each attempt, consistent results were obtained. It was difficult, however, to apply exactly the same amount of material to the surface each time, so the following practice was followed: the distance was measured at which each sample developed a pressure of, say, 18 dynes/cm., then a factor was used to calculate the results which would have been obtained if the same amount of material as in the first sample had been used.

Calculations of molecular weight, etc. were not made in the preliminary experiments, since mixtures of unknown composition were being used.

Measurement of Surface Potential.

When a metallic electrode is placed in the air close to the surface of water rendered conductive by salts, a potential difference between electrode and water may be observed. If a monolayer is spread on the water the potential difference is changed, and the change is defined as the surface potential of the monolayer.

To measure surface potential, the polonium air-electrode method was used. An electrode of radioactive polonium, covered with aluminium foil, was placed just above the surface so that the alpha radiation ionized the air gap between electrode and water. An Ag/AgCl electrode was used in the water. The normal potential (about 600 mV) was backed off with a battery and potentiometer. Changes in potential were indicated on an electrometer voltmeter (Vibron) and simultaneously recorded on paper by a pen recorder.

TABLE IV

Surface behaviour of extract W (red cell ghosts extracted with chloroform-methanol $2\frac{1}{2}$:1). The values of surface potential have been corrected for a standard quantity of extract.

Surface Potential	Distance in cm.				
	Without glucose		With glucose		
40 mV.	10·5 cm.	10·3 cm.	9·7 cm.	11·5 cm.	10·4 cm.
80	10·2	9·9	9·5	11·1	10·1
120	10·1	9·8	9·4	10·8	9·5
160	9·8	9·6	8·5	10·3	9·1
200	9·5	9·4	8·4	8·5	7·5
240	6·8	8·5	8·2	5·8	5·5
280	5·6	6·9	6·2	4·0	4·3
320	4·4	5·6	4·8		1·9
360		4·4			

Measurements of surface potential are usually used to study the degree of homogeneity of a film when it can exist, for instance, in either a gaseous or liquid phase (Harkins, 1949) or to follow a chemical reaction taking place at the interface (Rideal, 1948), but it may also be a measure of the dipole moment of the molecules forming the film.

The polonium electrode was placed at the end of the trough, near the glass slide. As the barrier was pushed towards it, the surface potential was seen to rise, either before, after or at the same time as the rise in surface pressure.

The results obtained for each sample were recalculated using the factors worked out from the surface pressure measurements, so that potential changes for a standard quantity of material were obtained. Experiments were performed (i) with 0·9% NaCl only in the trough, and (ii) after the addition of 1 g. glucose to the saline. The results were tabulated as shown in Table IV and plotted on graphs as shown in Fig. 6.

It may be seen from the diagrams of Fig. 6 that glucose has no effect on extract X, but may have some effect on W and Y.

Some measurements were made later on monolayers of lipids which were being used as standards in the development of the chromatographic techniques. Solutions of known concentrations of the lipid, preferably in cyclohexane which is not soluble in water and evaporates fairly rapidly, were applied to the cleaned surface of the saline in the trough from a micrometer syringe. It was thought that the presence of protein might stabilize the lipid and prevent it coalescing into hydrophobic aggregates: to this end, solutions of insulin in saline adjusted with HCl to pH 3

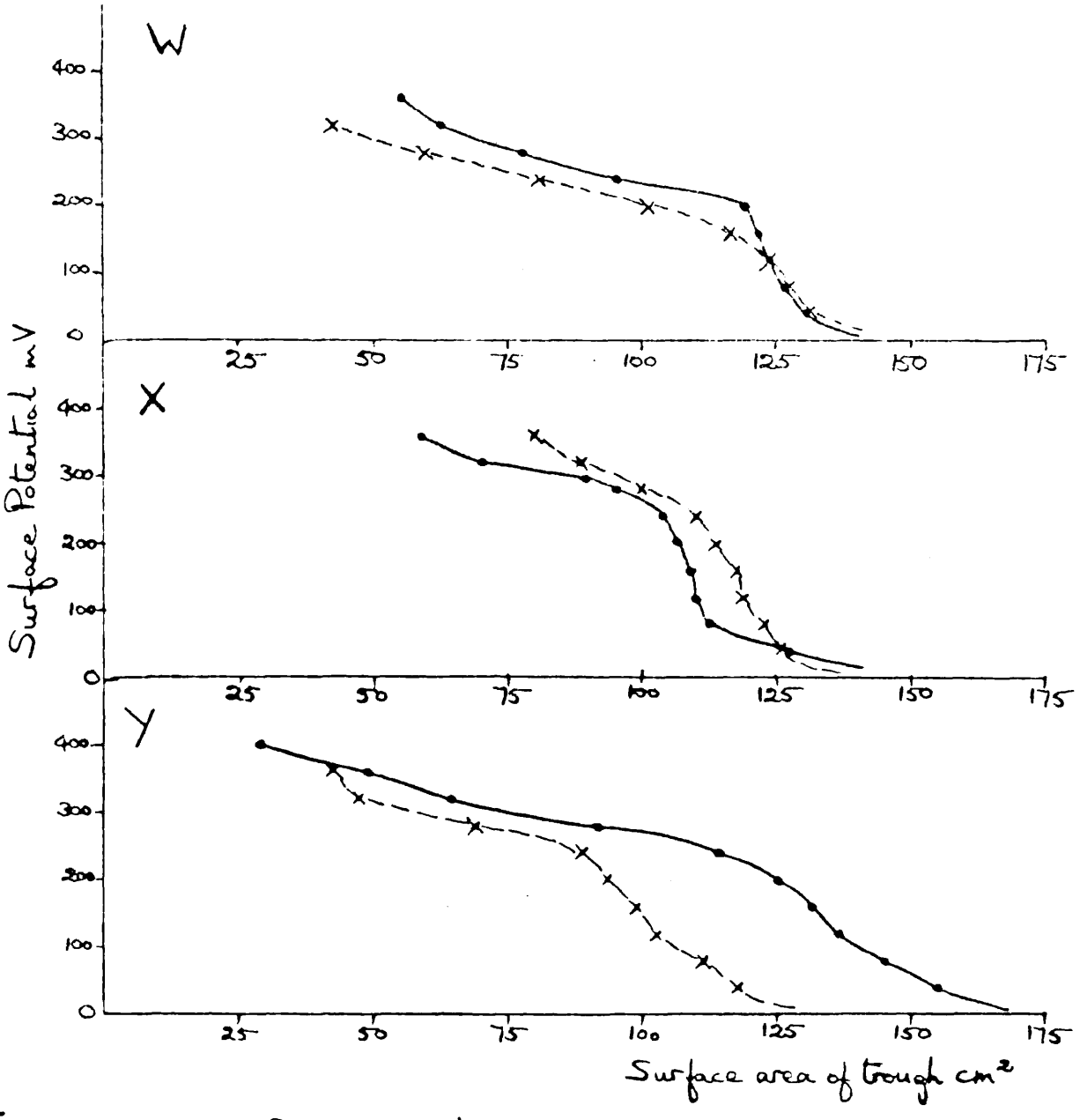


Fig 6. Surface Potentials of human red cell ghost extracts

W: extracted with chloroform-methanol 2½:1.

X: extracted with 3% ether in petroleum spirit.

Y: residue from X extracted with 40% ether in petroleum spirit.

●——● no glucose in substrate (mean of 2 results)
×——× glucose in substrate (mean of 3 results)

were also applied to the surface. Protein does not spread so rapidly as lipid, and some of it enters into solution in the substrate. After applying insulin, the surface was left undisturbed for 40 min. to allow the protein to distribute itself uniformly, but nevertheless the results obtained with insulin were rather variable. Each experiment was then repeated with 1 g. glucose dissolved in the substrate.

Figures 7 and 8 show the results obtained for cholesterol. The surface pressure curves, fig. 7, show the stabilising effect of insulin: the mixture of cholesterol and insulin in A exerts a higher pressure at all stages of compression than would have been predicted from the results for cholesterol and insulin alone. In the presence of glucose this effect is not so marked.

The surface potentials, shown in fig. 8, are not so easy to interpret, but the high potential achieved by cholesterol with insulin at 300 cm^2, that is before compression has begun, is probably also the result of higher stability, with the cholesterol molecules firmly aligned so that their charged groups are nearly all orientated in the same direction. It seems that the presence of glucose to some extent upsets this alignment process.

Fig. 9 shows the results obtained for monolayers of sphingomyelin and insulin. It may be seen that the presence of glucose made virtually no difference to the development of surface potential on compression.

Fig. 10 shows the results for a synthetic batyl analogue of phosphatidylethanolamine. The surface potential curves show not only the stabilising effect of insulin but also an interesting effect of glucose on the lipid alone.

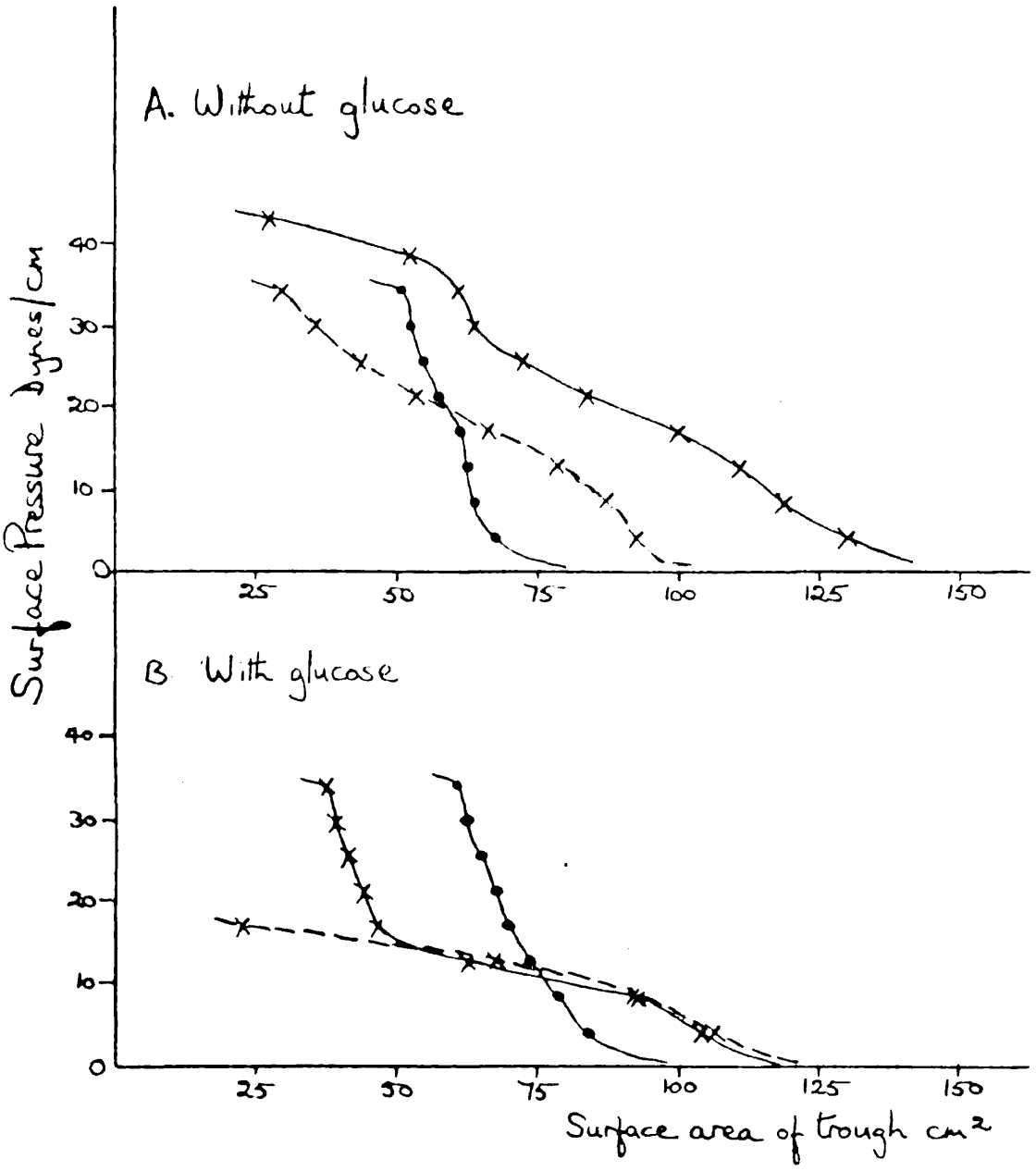


Fig 7. Surface Pressures of Cholesterol and Insulin Monolayers

A: Without glucose

B: With glucose

●——● 10µg cholesterol

x- - -x 16µg insulin

x——x 5µg cholesterol + 8µg insulin

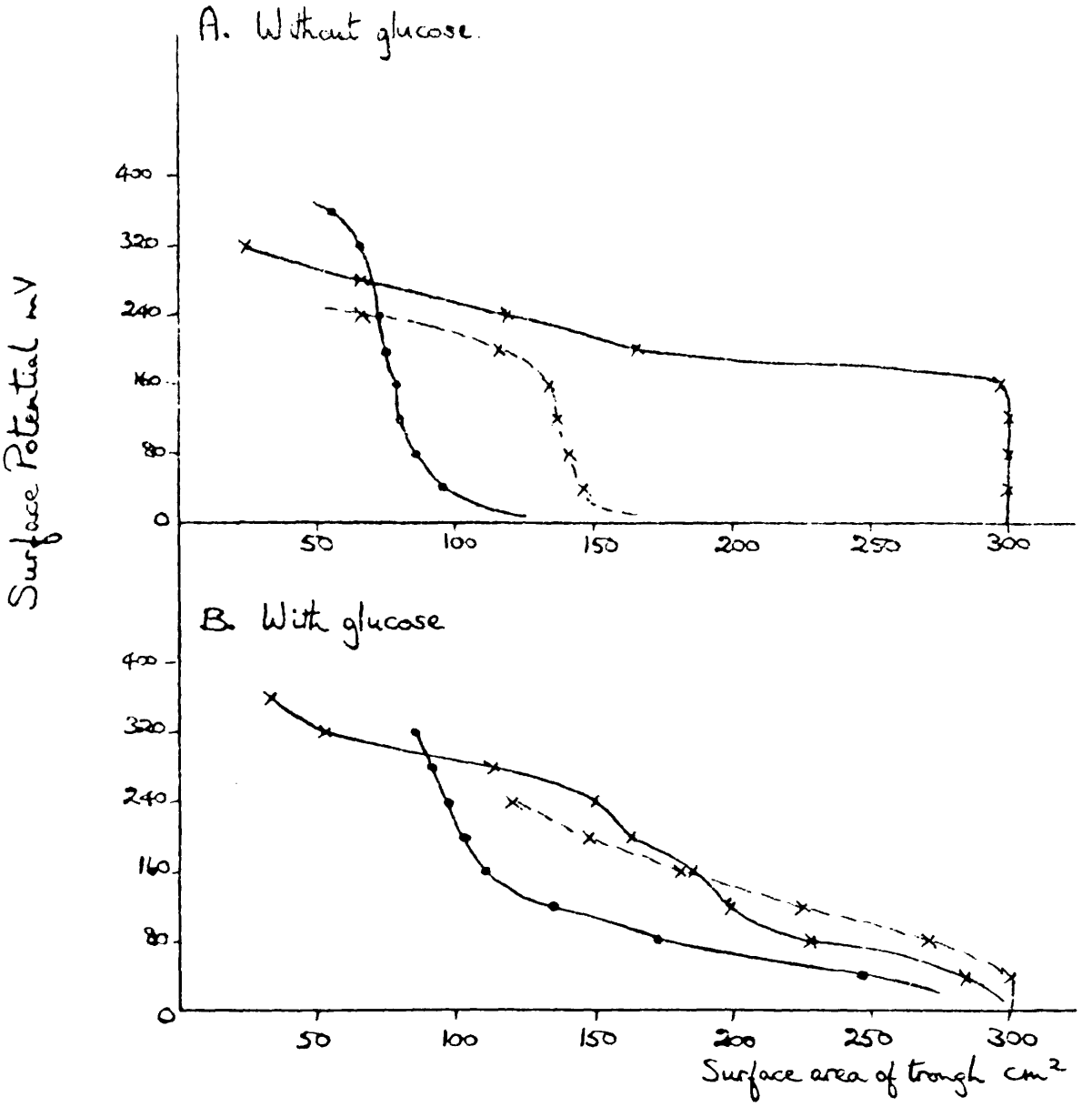


Fig 8. Surface Potentials of Cholesterol and Insulin Monolayers

A: Without glucose

B: With 1g glucose dissolved in substrate

●—● 10μg cholesterol in chloroform.

×– –× 16μg insulin in saline at pH3.

×—× 5μg cholesterol + 8μg insulin.

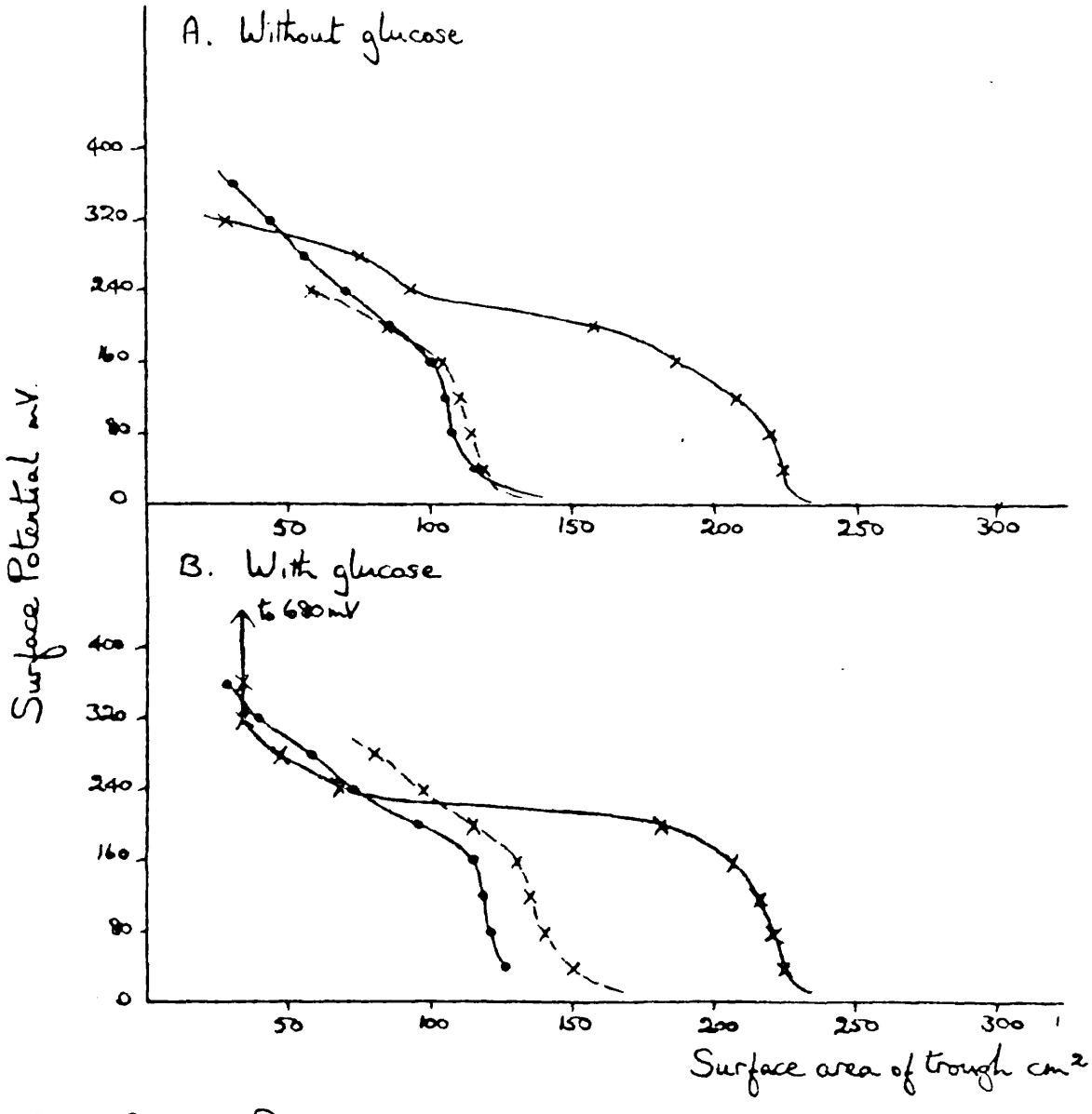


Fig 9. Surface Potentials of Sphingomyelin and Insulin Monolayers

A. Without glucose
B. With glucose, 1g, dissolved in substrate

●——● 13·5 µg sphingomyelin, in chloroform-methanol.
×--× 16 µg insulin in saline pH 3·8.
×——× 13·5 µg sphingomyelin + 16 µg insulin

Each curve is the mean of 2 results.

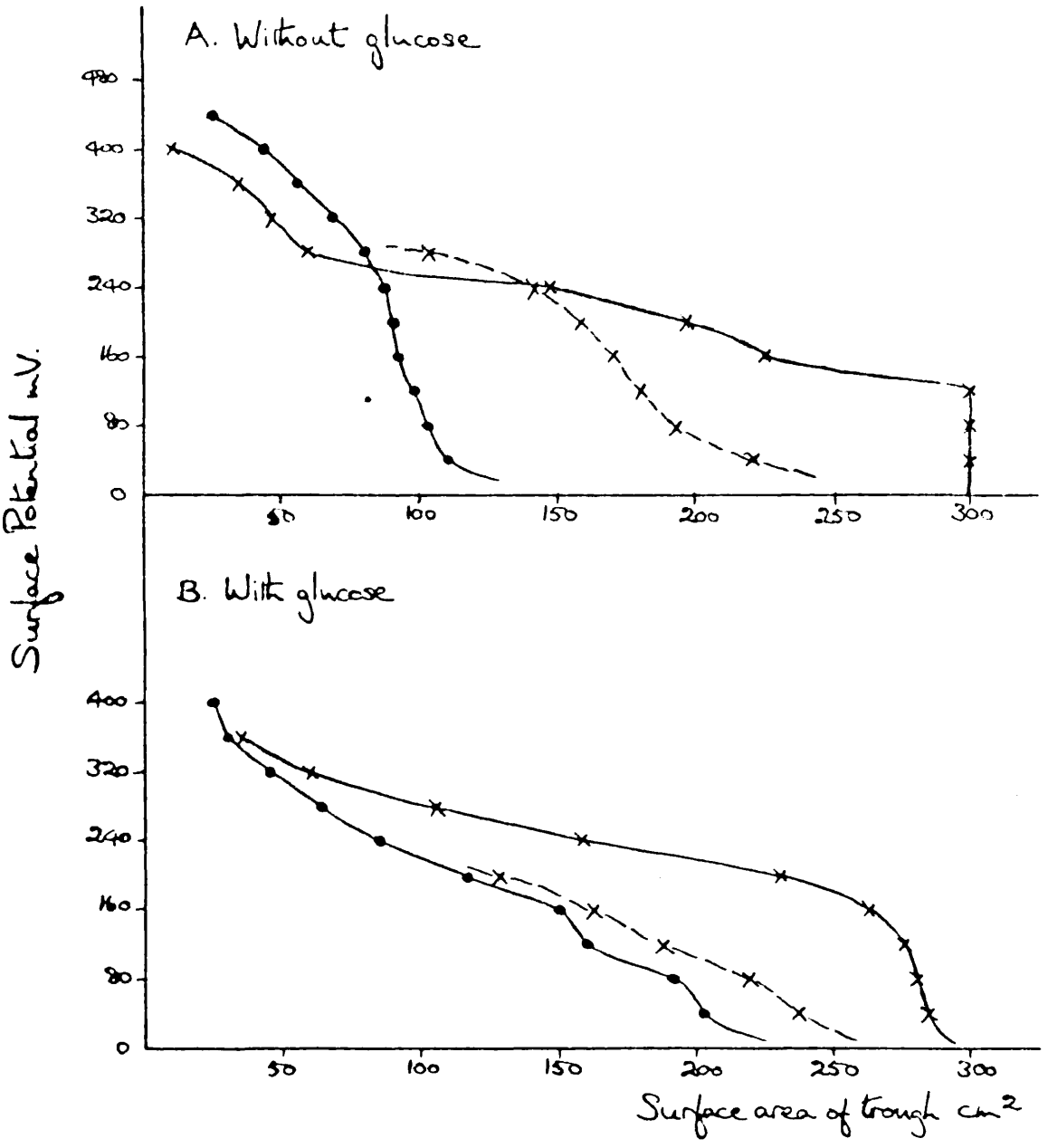


Fig 10. Surface Potentials of Phosphatidylethanolamine and Insulin Monolayers.

A: Without glucose

B: With 1g glucose dissolved in substrate

●—● 0·2ml butyl analogue of PE in chloroform-methanol-ethanol.

x--x 16μg insulin in saline pH 3

x—x 0·1ml PE + 8μg insulin.

Each curve is the mean of 2 results.

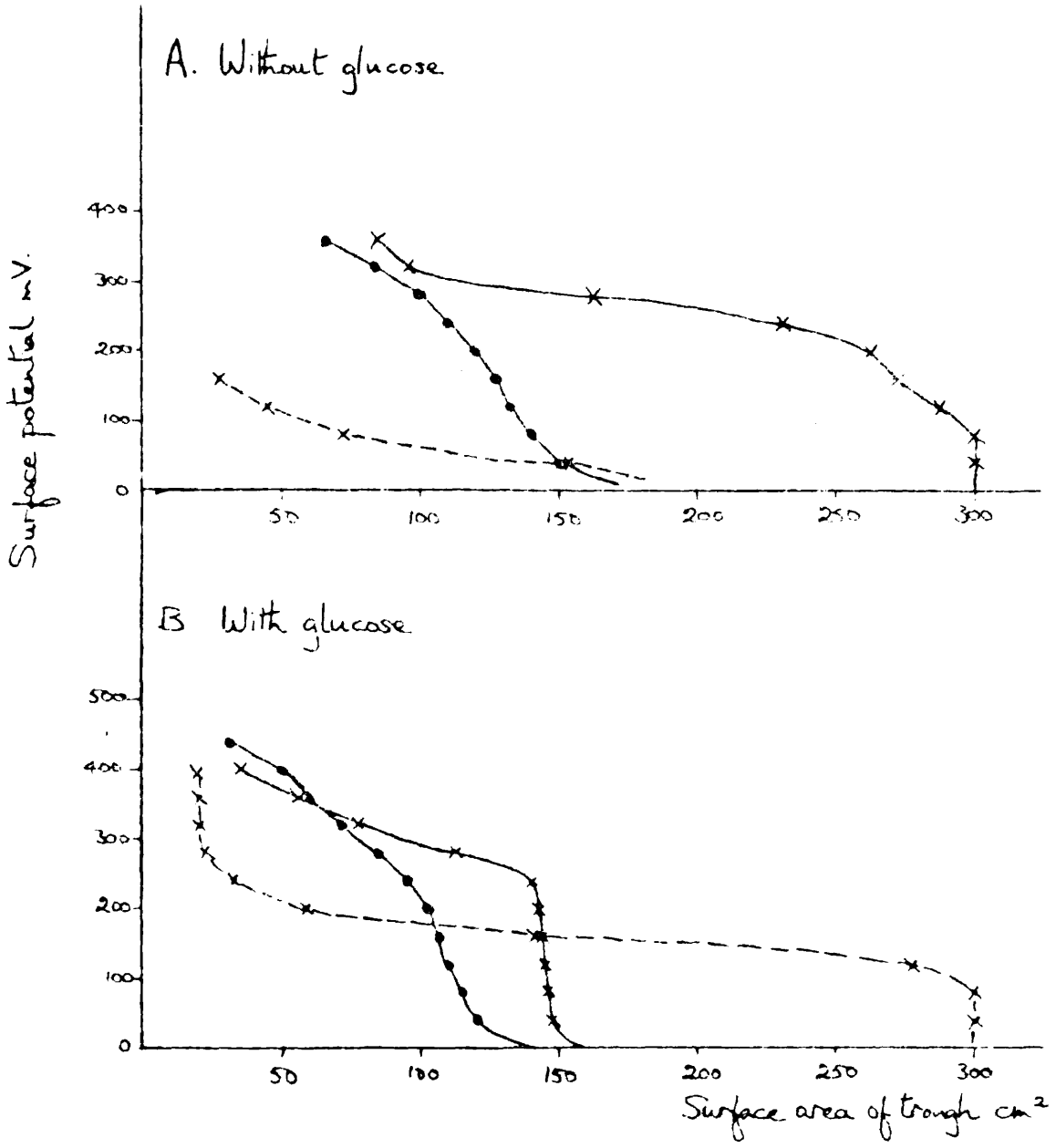


Fig 10A Surface Potentials of Lecithin and Insulin Monolayers.

A: Without glucose

B: With glucose, 0·5g, dissolved in substrate.

●——● Lecithin (2 results)

×——× Insulin, applied to surface, + lecithin.

×– –× Insulin, dissolved in substrate, + Lecithin.

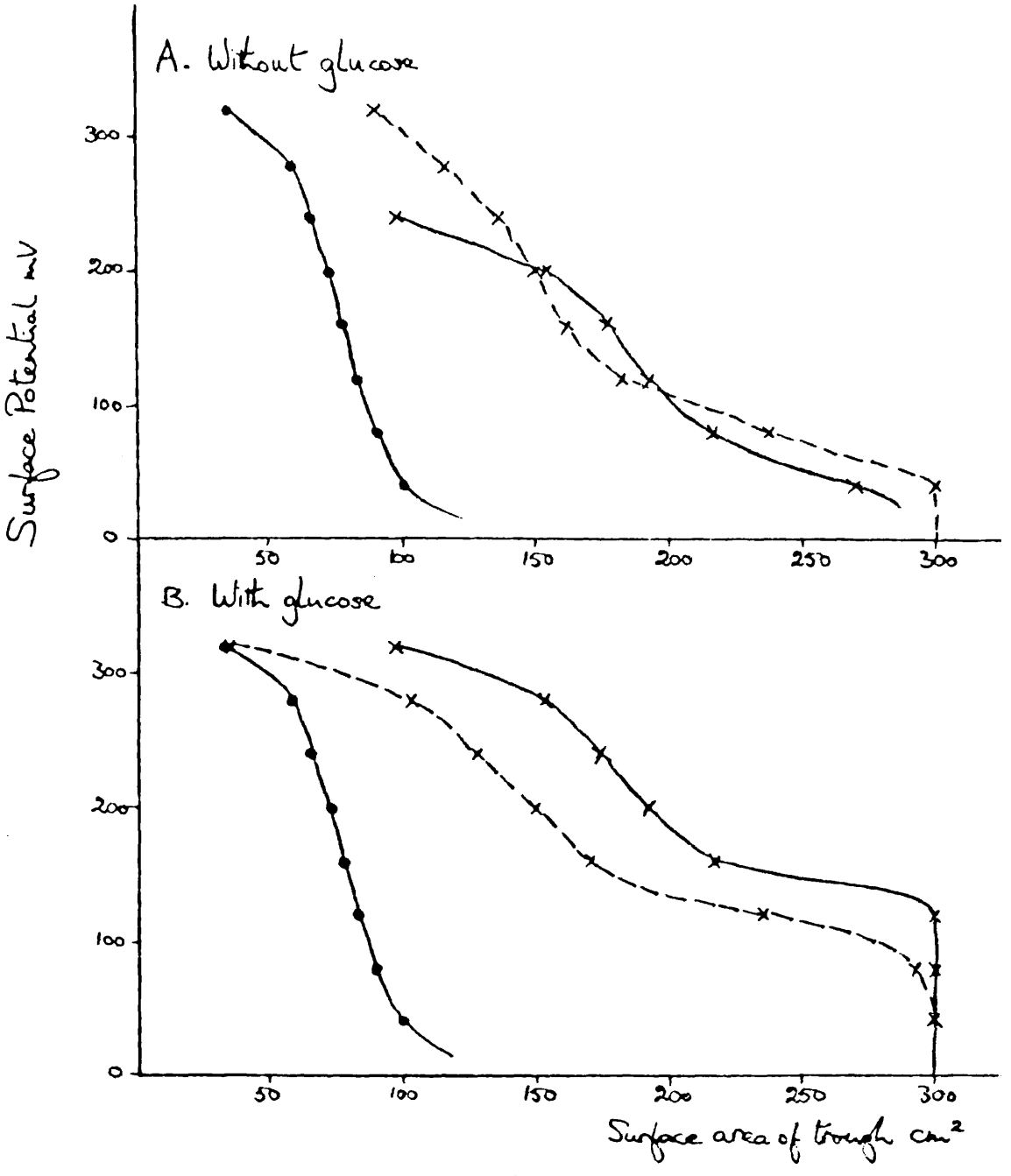


Fig. 11. Surface Potentials of Cerebroside and Insulin Monolayers.

A. Without glucose B. With glucose, 1g. dissolved in substrate

●——● 15µg cerebrosides.

×– –× 16µg insulin at pH 3.

×——× 15µg cerebrosides + 16µg insulin.

Each is the mean of 2 results.

Fig. 10A shows the results for egg-yolk lecithin, with a possibly interesting effect of glucose.

Fig. 11 shows the results for cerebrosides, where insulin appears to have some effect.

These results, though not valuable in themselves, show the sensitivity of the technique and its possibly valuable application to the study of reactions between lipids and sugars.

Preliminary Experiments on Labelling of Lipids.

The exploratory experiments described above appeared promising enough to encourage further preliminary experiments of a more ambitious nature. The methods of extraction used above were not completely satisfactory and were replaced by the two methods which will be described below.

In addition, an attempt was made to localise the proposed phospholipid membrane carrier by labelling it with radioactive glucose and with phloretin. R.A. glucose was chosen because of the ease with which small amounts may be detected. However, the reversible complex formed between glucose and the carrier molecule may be too unstable to withstand the extraction procedure. Phloretin is known to attach itself firmly to red cell membranes (LeFevre & Marshall, 1959), so should be easier to extract. The disadvantage is that phloretin may exert its inhibitory action on glucose transfer by combining not with the glucose site but at an adjacent point (Bowyer & Widdas, 1958). Nevertheless, an attempt was made to localise the lipids which do form associations with phloretin, by use of the UV spectrum of phloretin. R.A. glucose was also used in the presence of phloretin to see if the inhibitor affected the uptake of radioactive glucose.

1. Extraction with petroleum spirit/ether/alcohol.

Human erythrocytes were prepared from time-expired blood as described previously.

[^{14}C]-glucose (Universal labelling), specific activity 50-80 m.curies/m.mole, was obtained from the Radiochemical Centre, Amersham, Bucks. 1·48 mg. was made up to 10 ml. with saline buffer and stored at 4°.

Phloretin. The stock solution contained 1% phloretin in absolute alcohol. For the working solution, 0·275 ml. stock was made up to 10 ml. with buffered saline pH 7·4. In the mixtures used, this gave a final phloretin concentration of about 10^{-2} mM.

Solvent mixtures for extraction of ghosts were:-

5% ether in petroleum spirit.

50% ether in petroleum spirit.

20% ethyl alcohol in ether.

Procedure.

For the first stage of the extraction, two mixtures were made up:-

60 ml. ghost suspension.

2 ml. [^{14}C] glucose.

75 ml. 5% ether in petroleum spirit.

Either [P] 1 ml. phloretin solution,

or [S] 1 ml. buffered saline.

Each flask was stirred with a magnetic flea for 15 min. at room temperature, then left overnight at 4°. 50 ml. clear supernatant, containing the ether-petroleum spirit extract, was obtained from each; a little was put by for UV-spectroscopy and radioactive counting, and the remainder was evaporated to dryness under reduced pressure in an atmosphere of nitrogen at about 50° and weighed.

TABLE V

Results obtained from extraction of human red cell ghosts by:-

(1) 5% ether in petroleum spirit;

(2) 50% ether in petroleum spirit;

(3) 20% ethyl alcohol in ether.

Solvent	Dry Weight of 50 ml. extract (mg.)		Radioactivity of 50 ml. extract (c/m.)		Absorption difference 283mμ - 400mμ	
	S	P	S	P	S	P
1	39·0	69·9	1,325	550	0·125%	0·10%
2	50·2	41·8	5,875	4,425	0·06%	0·12%
3	116·4	47·7	10,500	11,600	0·15%	0·43%
Total	205·6	159·4	17,700	16,575		

S = without phloretin.

P = with phloretin.

For the second stage of the extraction, 60 ml. 50% ether in petroleum spirit was added to the ghost suspensions remaining in the flasks. The mixtures were magnetically stirred for 15 min. at room temperature, then left to stand for 15 min. 50 ml. clear supernatant was decanted off and treated as in the first stage.

For the third stage, 60 ml. alcohol in ether was added to the remains of the ghost suspensions in the flasks, stirred for 15 min. at room temperature and then left to stand for 15 min. 50 ml. clear supernatant was decanted and treated as before. The residue was cloudy and red, and probably contained mostly denatured haemoglobin.

Radioactivity measurements were carried out on duplicate 0·1 ml. samples of the supernatants on aluminium planchets, dried under a lamp to give a layer of lipid infinitely thin, and counted using a Geiger-Müller counter for ten minutes or until the total number of counts exceeded 100. Background radiation was subtracted from the result obtained and the counts per minute per 50 ml. supernatant calculated.

The UV spectra of the supernatants were recorded in the range 200mμ - 450mμ. Phloretin has peaks of absorption at 283 or 325 mμ, depending on pH and thus on whether it is in the enol or keto form (LeFevre & Marshall, 1959). In these experiments the value of absorption at 283mμ was recorded; 1% phloretin in alcohol, diluted with saline to give a 10^{-2} mM. solution, had an absorption of 0·12% at 283mμ.

The results obtained are shown in Table V. It may be seen that the presence of phloretin does not increase the amount of lipid nor the amount of radioactivity extracted. It is not possible to assess the

significance of the different distributions of weight and radioactivity in the different solvents since only one experiment was performed. The main bulk of the phloretin came out in solvent 3, that is alcohol in ether. This is where it would be expected on the basis of its known solubility in alcohol.

2. Extraction of Human Erythrocyte Ghosts with n-Butanol.

The higher alcohol n-butanol was chosen as an extracting solvent because it combined the power to split the phospholipids from their association with protein with good lipid solvent properties. It is only slightly soluble in water. Its main disadvantage is the high boiling point, which made evaporation to dryness of the lipid extracts a slow process.

Human erythrocyte ghosts were prepared from time-expired blood as described previously.

The solutions of phloretin and glucose were the same as used for the previous experiment.

Procedure.

The following mixtures were made up:-

75 ml. resuspended ghosts (from 1 pint blood).

2 ml. [^{14}C] glucose.

Either [P] 1 ml. phloretin solution,

or [S] 1 ml. buffered saline.

After a short interval allowed for equilibration, 75 ml. n-butanol was added.

The mixtures were stirred magnetically and allowed to stand for one hour.

TABLE VI

Results obtained from extraction of human red cell ghosts with n-butanol, and a comparison of the butanol, aqueous and solid phases.

Phase	Dry Weight of 50 ml. extract		Total Radioactivity in Phase		Absorption Difference 283mμ - 500mμ	
	S	P	S	P	S	P
Butanol	133 mg.	139 mg.	3.9×10^5 c/m.	2.6×10^5 c/m.	0.35%	0.43%
Aqueous			2.5×10^6 c/m.	2.4×10^6 c/m.	0.8%	1.0%
	Total wet weight					
Solid	14.9 g.	14.3 g.	5.5×10^4 c/m.	7.5×10^4 c/m.		

S = without phloretin.

P = with phloretin.

From each flask 50 ml. clear supernatant was obtained, of which 25 ml. was evaporated to dryness under reduced pressure, in a stream of nitrogen, at 50°, and weighed. 5 ml. was used for UV spectrometry and radioactivity measurements. The wavelengths chosen for the measurement of phloretin absorption were 283mμ and 500mμ. Duplicate 0·1 ml. samples were taken and dried on planchets for radioactive counting.

The residue was spun to separate distinctly the remaining butanol from the other phases. Both S and P mixtures gave the same volumes: 65 ml. aqueous layer and 20 ml. solid residue which had a wet weight of approximately 14·6 g. Radioactive measurements were taken of both phases, and the UV spectrum of the aqueous phase was recorded.

The results are shown in Table VI. It may be seen that the yield of lipid by extraction with butanol is similar to the total yield obtained with petroleum spirit/ether/alcohol, but that the yield of radioactivity is very much greater. Phloretin appears to be present in both butanol and aqueous phases. Its presence in the original mixture has little effect on the yield of lipid or radioactivity obtained.

Solubility of glucose in butanol.

The presence of glucose in the butanol extract to such a large extent could be due to three factors: the solubility of glucose in butanol (but this is known to be very low), the solubilisation of glucose by phospholipid (a property of phospholipids which has been discussed already), and the binding of glucose to a butanol-soluble lipid. In the hope of distinguishing between the last two, a portion of the butanol

extract was shaken with water and the radioactivity of the two phases was measured to find how much glucose had left the butanol in favour of the water.

The procedure was as follows: 3 ml. distilled water was added to 1 ml. butanol extract and the mixture was shaken for five minutes. On spinning, three layers separated out: a clear butanol upper layer, a slightly cloudy aqueous lower layer, and in between them an almost solid white layer which was actually an emulsion of butanol and water. 0·1 ml. samples of the butanol and water layers were taken and dried for radioactivity measurements. 0·3 ml. of the white layer was removed with some difficulty and made up to 3 ml. with absolute alcohol. 0·1 ml. samples were used. The results are given below, with the results of the original extraction for comparison.

Original extract	S	P
Butanol phase	789 c/m/0·1 ml.	517 c/m/0·1 ml.
Aqueous phase	3,886 c/m/0·1 ml.	3,720 c/m/0·1 ml.
Water added to butanol phase		
Butanol layer	200 c/m/0·1 ml.	353 c/m/0·1 ml.
Aqueous layer	138 c/m/0·1 ml.	72 c/m/0·1 ml.
White layer	195 c/m/0·1 ml.	190 c/m/0·1 ml.

On reshaking and respinning of the mixtures, very similar results were obtained.

It may be seen that, after addition of water to the butanol phase, the concentration of glucose remained higher in the butanol than in the

water layer, excluding simple solubility and suggesting that more than mere solubilisation of glucose by the phospholipids may be responsible. The difference between S and P is interesting and suggests that phloretin may be holding glucose back as well. The white layer, potentially interesting as a place where interfacial material might accumulate, showed a glucose concentration somewhere between the butanol and water concentrations.

The ultra-violet spectrum of phloretin.

Two properties of phloretin were investigated: its distribution between water and butanol and its tautomerism at different pH's.

For the first, an alcoholic solution of phloretin, 10 mg./ml., was diluted 2·75 ml. ⟶ 100 ml. with phosphate buffer. 1 ml. of this dilute phloretin was added to 5 ml. water and 5 ml. n-butanol in a tightly-stoppered tube, to give a concentration of 10^{-1} mM, and shaken for 5 min. On standing, the phases separated and their UV spectra were recorded against water of butanol in the reference cells. For comparison, a 10^{-2} mM phloretin solution in phosphate buffer was also recorded. The results are shown below:-

	Peak	Optical Density
Phloretin 10^{-2} mM in phosphate buffer	285mμ	0·12
Water phase of mixture	no distinct peak	0·03
Butanol phase of mixture	290mμ	0·32

It is clear from the results that most of the phloretin remains in the butanol phase.

To study the tautomerism of phloretin, mixtures of various pH were made up by addition to aqueous solutions appropriate quantities of sodium

bicarbonate or hydrochloric acid, and a comparison was also made between a solution in water and a solution in Krebs solution. The results are shown below:-

Phloretin solution	Peak mμ	Optical Density
1:50,000 in water (approx. pH 7)	290 mμ	1·1
1:50,000 in Krebs (approx. pH 8)	320	1·2
10^{-2} mM. in water (pH 4·0)	290	1·2
" " " " (pH 6·0)	285	1·1
" " " " (pH 7·0)	283	0·94
" " " " (pH 8·8)	320	1·5

These results agreed with those of LeFevre and Marshall (1959) and showed that the phloretin molecule is sensitive to pH, particularly in the range pH 7–8. The conclusion drawn by LeFevre and Marshall is that the peak at 284mμ signifies the presence of the ketonic form and the peak at 320mμ that of the enolic form.

Comparison of intact red cells with ghosts.

To compare the lipid and glucose yields from intact erythrocytes and ghosts, each was extracted with n-butanol using the same method and carrying both out simultaneously.

One pint human time-expired blood was washed as described previously and gave 200 ml. packed red cells. Another pint gave 60 ml. ghosts. The following extraction mixtures were made up:-

200 ml. red cells	60 ml. ghosts
1 ml. [^{14}C]-glucose solution	1 ml. [^{14}C]-glucose solution
250 ml. n-butanol	70 ml. n-butanol

The supernatants were treated as before and the following results obtained:-

	Red Cells	Ghosts
Total dry weight of lipid extract	254 mg.	276 mg.
Total R.A. counts	195,000 c/m	174,000 c/m

It may be seen that the yields of both lipid and glucose were similar, so in future experiments intact red cells were used.

A comparison was then made of intact red cells extracted with and without phloretin. They were extracted with n-butanol as before, except that the glucose used was non-radioactive. The following mixtures were made up:-

180 ml. packed red cells (from 1 pint blood),

220 ml. n-butanol,

150 mg. glucose,

either [P] 2 ml. phloretin (10^{-2} mM. final concentration),

or [S] 2 ml. buffered saline.

The yields of lipid were S: 273 mg; P: 234 mg.

Extraction of human red cells with chloroform-methanol 2:1.

A comparison was made between extracting human red cells with chloroform-methanol 2:1 and with n-butanol.

Packed red cells were obtained from human time-expired blood as described previously.

[^{14}C]-glucose was dissolved in water to give 5 μc/ml. It was added to the red cells before the extracting solvent, to give the glucose-carrier complex a greater chance of forming.

The following procedures were carried out:-

A. Chloroform-methanol 2:1. (Method of Farquar, 1962.)

50 ml. red cells and 1 ml. [^{14}C] glucose (5μc) were mixed and shaken well for 20 seconds, then poured slowly into 250 ml. methanol. 10 minutes after the start of the procedure, 500 ml. chloroform was added while continually swirling the contents of the flask. The mixture was stirred with a glass rod to prevent the formation of large lumps of denatured haemoglobin which trapped the other constituents of the red cells. The mixture was stood overnight at 4°.

B. n-Butanol.

50 ml. red cells and 1 ml. [^{14}C] glucose (5μc) were shaken for 20 seconds, then poured slowly into 500 ml. n-butanol, swirling continually and stirring with a glass rod. The mixture was stood overnight at 4°.

Mixture A was filtered through glass wool and the extract was evaporated down at 37° under reduced pressure in a stream of nitrogen, adding ethyl alcohol from time to time to prevent frothing. When the volume had been reduced to about 50 ml. the extract was filtered on paper, and the paper washed with chloroform and ethyl alcohol. The extract was dried further on the rotary evaporator until only water remained, then chloroform was added and the mixture spun. The chloroform phase, which contained about 60% of the radioactivity, was dried and weighed. The aqueous phase and emulsion were discarded.

The total radioactivity recovered in the chloroform-soluble lipid was 31,000 counts/min.

Mixture B was filtered through glass wool, and the extract was evaporated down under reduced pressure in a stream of nitrogen at 70-80°.

The total yield of lipid was 120 mg.

The total yield of radioactivity was 65,400 counts/minute.

From this experiment it was concluded that the yield of glucose from chloroform-methanol extraction, even taking into account the discarded aqueous phase, was not so good as that of the butanol extraction, and that for red cells the butanol extract is easier to handle.

In a very similar experiment using sheep red cells from the slaughter-house, adding 5 μc. glucose to 50 ml. red cells, the yield of radioactive glucose was as follows:-

Chloroform-methanol 2:1 : 5,500 counts/min./50 mg. lipid.

Butanol : 17,400 counts/min./50 mg. lipid.

Extraction of freeze-dried human red cells.

Red cells were prepared from human time-expired blood. They were mixed with [^{14}C] glucose and freeze-dried as follows:

50 ml. red cells and 1 ml. [^{14}C] glucose (5 μc/ml.) were mixed for 20 seconds in a round-bottomed flask. Half the quantity was then poured into a second round-bottomed flask and both were plunged into a bowl of Drikold-methylated spirit, rotating the flasks continually until an even frozen layer of red cells was obtained all round the flasks. They were then attached to the rest of the apparatus and evacuated by a vacuum motor pump. Ice formed on the outside of the flask, then after one hour disappeared: the red cells were then dry. The vacuum pump was released very slowly.

250 ml. n-butanol was added to each flask, shaken and stood overnight at 4°.

The butanol extracts were decanted and evaporated to dryness as before. The yields of lipid were (1) 63·8 mg.; (2) 46·6 mg.; total yield 110·4 mg. per 50 ml. red cells.

The lipid from (1) was eluted on silica-impregnated filter paper with chloroform-methanol for 36 hours, by descending chromatography, to rid it of impurities (Biezenski, 1962). The yields of radioactive glucose were as follows:-

(1) 700 counts/min./63·8 mg.

(2) 840 counts/min./46·6 mg.

It may be seen by comparing these results with earlier ones that the extracts of freeze-dried red cells gave a good yield of lipid but a low yield of glucose. It is possible that only free glucose remained behind after the butanol extraction: for instance, the phospholipids may need to be slightly wet to exert their solubilising effect on sugars. But it is also possible that the hypothetical glucose-carrier complex, which would probably be partly hydrophilic, would not be extracted by butanol under these conditions. In view of this, freeze-drying was not used in further experiments. The method of purifying the extract from non-lipid contaminants was not used either, since the column chromatography used subsequently to fractionate the lipids also separated lipids from contaminants.

General Procedure for Extraction of Red Cells.

These preliminary experiments on extraction of lipids from red cells, with or without radioactive glucose, indicated n-butanol as the best extracting solvent, to be used in a volume a little larger than the volume of red cells. In later experiments the temperature at which the extract

was evaporated down was kept at 37° or below, which made the process slower but still possible.

The results with phloretin did not suggest that it was valuable as a marker for the membrane carrier, nor was the ultra-violet method of estimating it very satisfactory. In later experiments other inhibitors were tried, notably 2:4 dinitrofluorobenzene (DNFB).

These preliminary experiments also made apparent the speed with which lipids deteriorated unless special precautions were taken. In future experiments, red cells and mixtures were kept as cold as possible and nitrogen was bubbled through mixtures and extracts before storing.

Extraction of Sheep Placental Tissue.

As a preliminary experiment, sheep placental tissue was extracted successively with acetone, to obtain neutral lipids, with chloroform-methanol 2:1 for phospholipids, and with chloroform-methanol-conc.HCl for proteolipids.

220 g. cotyledons from the placenta of a sheep were removed three hours after injection into the umbilical vein of 1 m.curie [^{14}C] fructose, as described earlier. They were ground with 750 ml. acetone in a Waring blender for 2 min., then the mixture was filtered on a Buchner funnel, washed with a further 50 ml. acetone, and the filtrate was evaporated to dryness as described for the red cell extracts.

The residue was put back into the Waring blender with 600 ml. chloroform-methanol 2:1, mixed for 20 seconds and washed into a flask with a further 50 ml. chloroform-methanol 2:1, and left to stand overnight at 4°.

The chloroform-methanol extract was filtered and evaporated to dryness as before, and the residue was further extracted by mixing it with 500 ml. chloroform-methanol-conc.HCl 200:100:1. This extract was filtered and dried as far as possible. It was a dark brown solution which, on evaporation, formed little colourless sheets of material and a dark sludge, part of which was soluble in chloroform-methanol, part in ether and part of which remained in an insoluble deposit.

Samples of each extract were taken for radioactive counting. The results are shown below:-

		Weight	Radioactivity
Acetone extract	(Dry)	1·2420 g.	480 c/m/0·1 ml.
Chlor-meth. extract	(Dry)	1·5110 g.	1,200 c/m/0·1 ml.
Chlor.-meth.-HCl extract	(Wet)	27 g.	186 c/m/0·1 ml. (chlor.-meth. sol.)
			0 c/m/0·1 ml. (ether sol.)
			6 c/m/0·1 ml. ("deposit")
			12 c/m/0·1 ml. ("membranes")

As a result of this preliminary experiment, chloroform-methanol 2:1 was used as the extracting solvent in all later experiments, and was found to bring into solution both phospholipids and neutral lipids. On standing, the chloroform and aqueous phases separated, with the extracted material in the middle. The chloroform phase was then removed and evaporated down at 37^{o} or below, under reduced pressure in a stream of nitrogen.

In an attempt to overcome the difficulties of evaporating down a wet chloroform extract without excessive frothing, and partly as an attempt to separate phospholipids from neutral lipids, the method of precipitating phospholipids in cold acetone was used (Hanahan, Dittmer & Warashina, 1957).

When the volume of the extract had been considerably reduced, a large excess of cold acetone was added and the flask was stood overnight at 4^{o}. The precipitated phospholipid, which accounted for about one-quarter of the total lipid, was separated from the supernatant by decanting and spinning in the refrigerated centrifuge. The supernatant was then evaporated to dryness, taking precautions against frothing. Subsequent fractionations on a silicic acid column showed that the separation of phospholipids by this method is not satisfactory: some phospholipid remained behind in the acetone-soluble lipids and some neutral lipids came down with the phospholipid. This is presumably because of the mutual solubility of so many of the lipids.

Having described the way in which lipids were extracted from human and sheep red cells and from sheep placental tissue, the separation of these lipids will now be discussed and the methods used to identify them.

PART 2 - SEPARATION OF LIPIDS BY CHROMATOGRAPHIC TECHNIQUES.

Adsorption chromatography was discovered by Tswett in 1903 when he separated plant pigments by pouring a solution of them in petroleum spirit onto a column of calcium carbonate, whereupon different pigments travelled different distances because of their different adsorptions to calcium carbonate.

The idea was carried a stage further when Martin & Synge (1941) introduced partition chromatography. Here the substances were first adsorbed onto columns of wet silica gel, then eluted with organic solvents. The "mobile" organic phase passed over the "stationary" aqueous phase, so that the substances were partitioned between the two phases. Substances of different partition coefficient were thus eluted at different speeds. From this technique sprang paper chromatography, modern column chromatography and thin-layer chromatography.

The theory of adsorption chromatography has been discussed and reviewed frequently (Smith, 1960; Randerath, 1963). For a mixture of substances to be successfully separated on a chromatographic column, all the material should be reversibly bound to the surface of the adsorbent to a greater or lesser extent, and the eluting solvents used should be powerful enough to free the materials from their adsorbed state. Adsorbents are usually oxides, as in the case of silica, and they preferentially adsorb polar or polarisable compounds by binding them by electrostatic forces onto the crystal lattice.

In practice, this means that in a mixture of lipids the polar phospholipids such as lecithin and sphingomyelin are firmly adsorbed to the silica and require a high proportion of the polar solvent methanol in the eluting solvent to free them from the column. Neutral lipids such as triglycerides, on the other hand, may be completely eluted by the non-polar solvent chloroform.

Column chromatography has the advantage over paper chromatography that larger amounts of material may be thus separated. It also has the disadvantage that separation of lipids is usually not complete (Marinetti, 1962), so that more than one fractionation or procedure may be necessary to obtain a pure substance. For phospholipids the ideal combination now appears to be silicic acid column chromatography followed by thin-layer chromatography (T.L.C.), which gives a clear separation of small amounts of material.

Trappe (1940) was the first to use silicic acid columns for the separation of lipids by elution with chloroform and then with methanol. Lea et al. (1955) applied the method to the separation of the two major constituents of egg yolk phospholipid. Hanahan, Dittmer & Warashina (1957) used a series of chloroform-methanol mixtures and successfully separated all the main classes of phospholipids from liver and yeast. This is the method now in general use for the separation of lipids from many tissues, including red cell and plasma lipids (Hanahan, Watts & Pappajohn, 1960). The methods used in the present experiments were based on this work.

Thin-layer chromatography was introduced by Kirchner, Miller & Keller (1951) and by Stahl (1956) and has been used extensively for chemical analysis. It was introduced fairly recently for the separation of phospholipids and it rapidly gained favour as a quick and efficient method of separating small quantities (of the order of 10 μg.) of lipid material. (See, for instance, Vogel & Zieve, 1962.)

Some preliminary experiments on the use of silicic acid columns for the fractionation of red cell lipids will be described in this section. The techniques of thin-layer chromatography were developed for red cell and placental lipids a little later in the same laboratory by Ann C. Dawson, Judith M. Spencer and Mary L. Forsling, and the methods evolved will be described later.

Silicic Acid Column Chromatography.

Fractionation of Red Cell Lipids from early Extractions.

Lipid extracts were obtained in the experiments described in the first part of this chapter and were pooled into the following groups:-

Ghost extracts (1) with phloretin,
(2) without phloretin.

Intact red cell extracts (1) with phloretin,
(2) without phloretin.

Silicic acid: "silica gel suitable for chromatography" (May & Baker) was washed with chloroform–methanol 1:1, then finally with methanol on a Buchner funnel, and dried in an oven for 12 hours at 110° to activate it fully.

Columns: a variety of glass tubes were used, of various capacities, and they gave very similar results when used with the same proportion of silicic acid : lipid : eluting solvent. The larger ones had a sintered glass disc at the bottom, the smaller ones had a drawn-out tip and a glass-wool plug. Either there was room in the tube for the silicic acid and for a fair volume of eluting solvent, or there was fitted a reservoir for eluting solvent at the top. There was also an inlet for nitrogen, and the apparatus was air-tight so that a positive nitrogen pressure could be applied to accelerate the flow of solvent through the column.

2–10 mg. lipid (dry weight) per gram silicic acid gave good separation on the columns.

Procedure: the amount of silicic acid required was calculated, weighed, mixed with chloroform to form a slurry, poured into the tube and packed down with more chloroform until the surface of the column reached a constant level. Without allowing the level of solvent ever to fall quite as low as the top of the column, the lipid mixture, dissolved in a little chloroform, was poured onto the column and washed down with small portions of solvent (1). The fractionating solvents listed below were run through the column, allowing the level of the first to almost reach the top of the column before adding the second, and the eluent fractions were collected in flasks.

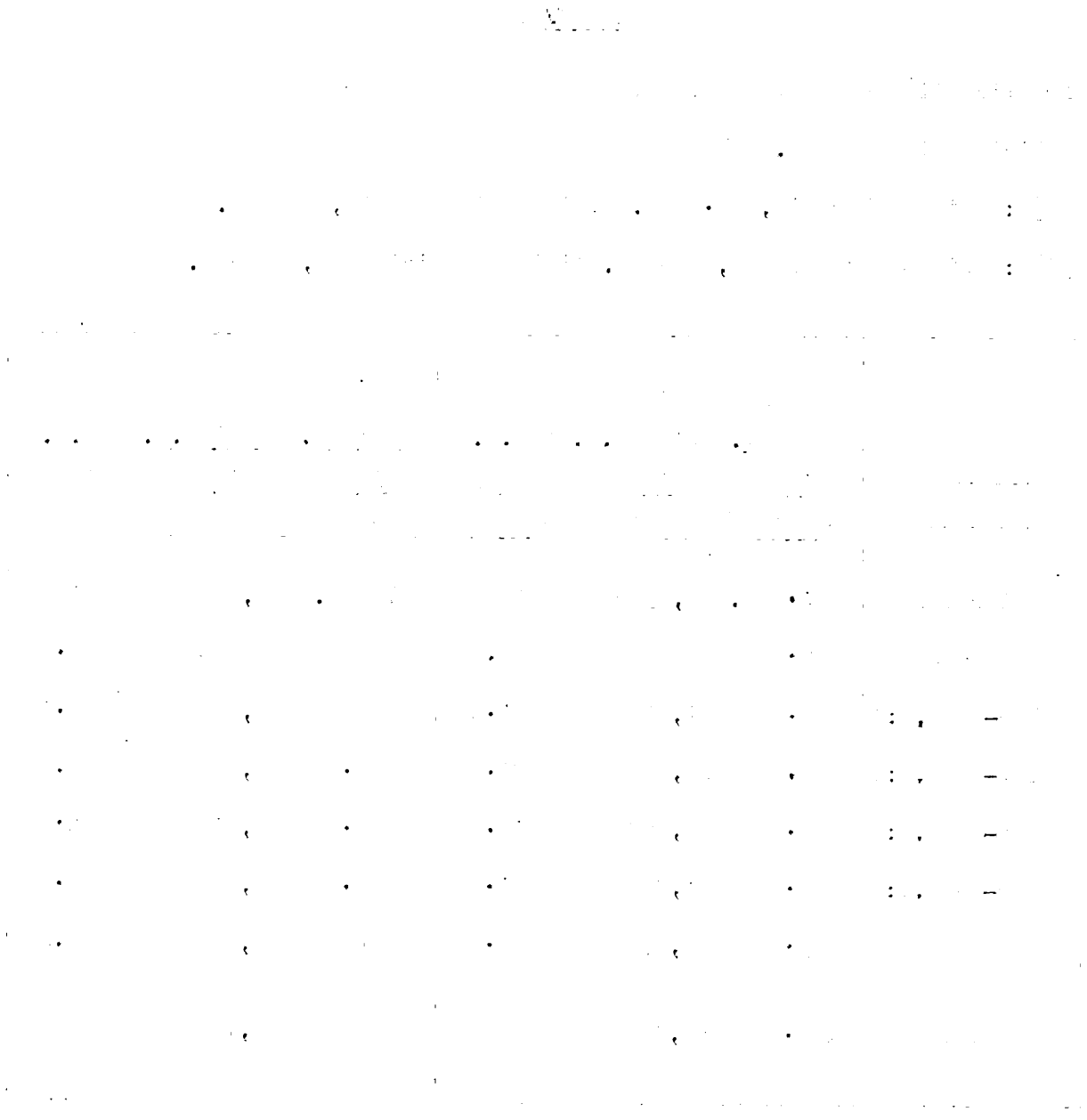


TABLE VII

Fractionation of pooled butanol extracts of human red cell ghosts on silicic acid columns.

[P] : with phloretin, 83·8 mg. lipid containing 44,900 c/m.

[S] : without phloretin, 286 mg. lipid containing 58,800 c/m.

Eluting Solvent	[P]			[S]		
	Total Wt. in Fraction	Total R.A. in Fraction	% R.A. in Fraction	Total Wt. in Fraction	Total R.A. in Fraction	% R.A. in Fraction
Chloroform	24·2mg.	18,390c/m	43%	174mg.	30,600c/m	54%
Chloroform	2·0	300	1.0	0	850	1·5
Chlor-meth.9:1	36·0	1,940	4·5	0	3,600	6·3
Chlor-meth.2:1	3·9	10,360	24·0	19·2	13,000	23·0
Chlor-meth.1:2	32·0	8,320	19·0	5·6	3,640	6·4
Chlor-meth.1:9	2·8	1,930	4·5	3·2	2,600	4·5
Methanol	5·0	1,570	4·0	0	2,900	5·1
Total	105·9	42,910		202	57,190	

(1) 250 ml. chloroform.

(2) 250 ml. chloroform.

(3) 100 ml. chloroform–methanol 9:1.

(4) 100 ml. chloroform–methanol 2:1.

(5) 100 ml. chloroform–methanol 1:2.

(6) 100 ml. chloroform–methanol 1:9.

(7) 250 ml. methanol.

The volume of each fraction was measured, then a 25 ml. sample evaporated to dryness, at reduced pressure, in a stream of nitrogen at 37°. The dry lipid was dissolved in 1 ml. chloroform or 1 ml. methanol. The 0.1 ml. samples were taken for radioactivity measurements and the rest was used for UV spectrometry.

Pooled butanol extracts of red cell ghosts, with and without phloretin, were fractionated on silicic acid columns and the results are shown in table VII. It may be seen that the recoveries of dry weight were erratic because of the inaccuracies of weighing such small amounts. The recoveries of radioactivity, however, were 98% for [P] and 95.5% for [S], and were probably accurate.

Most of the radioactivity came out in the first chloroform fraction and in the chloroform–methanol 2:1. In the phloretin extracts there was also a high proportion in the chloroform–methanol 1:2 fraction. This may be due to phloretin, which is more soluble in methanol than chloroform, holding some of the glucose back in the early fractions.

Pooled butanol extracts of intact red cells were fractionated in the same way. The glucose added was not radioactive. The dry weights obtained were rather erratic.

The results obtained from the UV spectrometry and from the monolayer studies were not very useful because of the poor results from the weighings, but some general conclusions were drawn from them. In particular, the peaks of absorption shown by the different fractions showed that different components or mixtures of components were present in each fraction, and these presumably corresponded with the findings of earlier workers using the same elution scheme. At that time, the reference compounds necessary to check their identity were not available. Differences were obtained between [P] and [S] extracts, and between ghost and red cell extracts, but their significance was unknown.

Surface-pressure-area curves were drawn for monolayers of each fraction: the chloroform-methanol 9:1 and 1:9 fractions showed a difference between the [S] and [P] extracts, but the others showed none.

Fractionation of freshly-extracted red cell lipids.

The experiment above was repeated using a fresh butanol extract of human red cells, taking greater precautions over the weighing procedures, and better results were obtained. A large volume of red cells was used in the hope that a carrier component present only in small proportions would be extracted in sufficient quantity to be located. The following mixtures were made up:-

[S] 880 ml. red cells (from 5 pints human blood ex-blood bank).
10 ml. [^{14}C]-glucose containing 25 μc. (total 4·2 x 10^6 cnts./min.).
880 ml. n-butanol.

The glucose and red cells were mixed together for 30 seconds before the addition of butanol, then shaken by hand for 15 min. The

TABLE VIII

Fractionation of human red cell butanol extracts on silicic acid columns.

[P] : with phloretin; [S] : without phloretin.

No. of fraction	Eluting Solvent	[S] Dry Wt.in fraction (mg)	[S] Counts in fraction (c/m)	[S] % counts	[P] Dry Wt.in fraction (mg)	[P] Counts in fraction (c/m)	[P] % counts
1	Chloroform	824	12,200	52·0	1,090	8,800	58·5
2	Chloroform	1·0	330	1·4	67·1	920	6·1
3	Chlor–meth. 9:1	2·8	3,070	13·0	2·7	550	3·7
4	Chlor–meth. 2:1	49·9	6,390	27·2	31·9	2,650	17·7
5	Chlor–meth. 1:2	46·5	480	2·1	46·6	890	6·0
6	Chlor–meth. 1:9	49·1	590	2·6	29·1	640	4·3
7	Methanol	36·0	550	2·4	26·1	650	4·3
Total		1,009 mg	23,610 c/m		1,294 mg	15,100 c/m	

mixture was left to stand for 15 min., then the butanol layer was decanted and the remainder spun to obtain the rest of the butanol.

Butanol extract recovered: 580 ml.

Total dry weight: 1·1036 g.

Total radioactivity: $1{\cdot}2 \times 10^5$ counts/min.

Recovery of radioactivity: 2·85%.

The lipid was dissolved in 8 ml. chloroform, stored at -5° and fractionated 6 days later.

[P] 720 ml. red cells (from 5 pints human blood ex-blood bank).

10 ml. [^{14}C]-glucose, 25 μc., total $4{\cdot}8 \times 10^6$ counts/min.

10 ml. phloretin, 0·275 mg./ml.

640 ml. n-butanol.

The procedure was the same as above, except that the mixture was strained through muslin before the butanol layer was allowed to settle.

Butanol extract recovered: 540 ml.

Total dry weight: 0·9072 g.

Total radioactivity: $1{\cdot}2 \times 10^5$ counts/min.

Recovery of radioactivity: 2·5%.

The lipid was dissolved in 8 ml. chloroform, stored at -5° and fractionated 5 days later.

The two lipid extracts were fractionated on silicic acid columns, using the same eluting solvents as before. The results obtained are shown in table VIII.

TABLE IX

Refractionation of fractions 3, 4 & 5 of butanol-extracted human red cells separated on silicic acid columns.

[S] 98 mg. containing approximately 9,000 c/m.

[P] 81 mg. containing approximately 4,000 c/m.

No. of fraction	Eluting Solvent	[S] Dry Wt.in fraction (mg)	[S] Counts in fraction (c/m)	[S] % counts	[P] Dry Wt.in fraction (mg)	[P] Counts in fraction (c/m)	[P] % counts
1	Chloroform	40·7	3,880	40·6	36.6	720	17·9
2	Chlor-meth. 19:1	33·4	640	6·7	46.5	590	14·8
3	Chlor-meth. 12:1	13·0	1,870	19·6	34·4	930	23·3
4	Chlor-meth. 9:1	62.0	1,360	14·3	25·6	390	9·9
5	Chlor-meth. 7:1	32·3	500	5.2	39·7	330	8.2
6	Chlor-meth. 6:1	87·9	600	6.2	40·1	900	22·5
7	Chlor-meth. 1:9	11·7	710	7·4	4·4	130	3·3
Total		281 mg	9,560 c/m		267·3 mg	3,990 c/m	

It may be seen that in each case there was probably overloading of the column so that too much material was eluted in the chloroform fraction, and this probably explained the high percentage of radioactivity recovered in this fraction. However, a considerable proportion was also recovered in chloroform–methanol 9:1 and 2:1 in the case of the [S] extract, and with chloroform–methanol 2:1 and 1:2 in the case of the [P] extract.

In order to obtain the glucose-containing materials in a more purified form, fractions 3, 4 and 5 were pooled and refractionated, and fraction 1 was also refractionated. The results are shown in tables IX and X.

It may be seen that in the refractionation of 3, 4 and 5 the recoveries of dry weight were too high. This could have been due to fine particles of silicic acid being eluted, and in future experiments this was avoided by more thorough washing of the silicic acid when in the column with both chloroform and methanol. The radioactivity as well as the weight was widely distributed, showing that the samples originally contained a mixture of several components. The interesting fractions were 1, 3 and 4, that is those eluted with chloroform and chloroform–methanol 12:1 and 9:1. In view of this, future extracts were fractionated by eluting first with chloroform–methanol 9:1 to obtain all this high-activity material in one fraction.

In the refractionation of fraction 1 of the [S] series, eluting solvent mixtures were made up with hexane, benzene and ether, according to the scheme of Barron and Hanahan (1958).

The results, set out in table X, show that the separation was not satisfactory for the purposes of this investigation, since the only

TABLE X

Refractionation of fraction 1 of butanol-extracted human red cells, in the absence of phloretin.

Eluting Solvent	Dry Wt. in fraction (mg.)	R.A. counts in fraction (c/m)	% R.A. in fraction
Hexane	498.6	13,960	93.5
15% benzene in hexane	5.6	590	3.9
5% ether in hexane	27.6	250	1.7
20% ether in hexane	7.4	0	0
50% ether in hexane	155.6	0	0
90% ether in hexane	55.3	120	0.8
Total	750.1	14,920	
Total put on column	742 mg.	11,000 c/m	

fraction to separate in any quantity from the hexane-eluted lipid was that in 50% ether/hexane, which contained no radioactivity at all.

Elution of Glucose from a Silicic Acid Column.

It was desirable at this stage to know how glucose behaved on the silicic acid column under the fractionation conditions being used, when in the free state, or when conditions allowed some solubilisation but probably no complexing with the phospholipids.

First, a suspension of D-glucose in chloroform containing 1·5 mg. was put on the column and eluted with the solvents used in earlier experiments. The fractions were dried, taken up in water and estimated for glucose by the glucose oxidase method (Huggett & Nixon, 1957), which has been shown to be sensitive and accurate for small quantities of glucose. The results are shown below.

Solvent	Glucose recovered
350 ml. chloroform	$0{\cdot}8 \times 10^{-2}$ mg.
100 ml. chlor-meth 9:1 100 ml. chlor-meth 2:1	$15{\cdot}8 \times 10^{-2}$ mg.
100 ml. chlor-meth 1:2	$30{\cdot}4 \times 10^{-2}$ mg.
100 ml. chlor-meth 1:9	$19{\cdot}1 \times 10^{-2}$ mg.
100 ml. methanol	$2{\cdot}7 \times 10^{-2}$ mg.
Total	0.688 mg.
Glucose put on column	1·5 mg.
Recovery of glucose	46%

TABLE A1

Fractionation of lipid with added glucose.

A. Red cell lipids from middle fractions of [S] series pooled with addition of 1·8 mg. glucose.

Solvent	mg. glucose	% glucose	% radioactivity
200 ml. chloroform	$0·4 \times 10^{-2}$	1·84	3·37
100 ml. chlor-meth.19:1	$0·45 \times 10^{-2}$	2·08	2·36
100 ml. chlor-meth.12:1	$0·60 \times 10^{-2}$	2·78	19·9
100 ml. chlor-meth. 9:1	$0·30 \times 10^{-2}$	1·38	19·2
100 ml. chlor-meth. 7:1	$0·20 \times 10^{-2}$	0·92	16·4
100 ml. chlor-meth. 5:1	$0·70 \times 10^{-2}$	3·22	8·9
100 ml. chlor-meth. 3:1	$0·90 \times 10^{-2}$	4·17	8·2
100 ml. chlor-meth. 1:9	$18·0 \times 10^{-2}$	83·52	22·8
Total	$21·55 \times 10^{-2}$		
Recovery of glucose	12%		

B. Red cell lipids from early fractions of [S] series pooled with addition of 2·6 mg. glucose.

Solvent	mg. glucose	% glucose	% radioactivity
200 ml. chloroform	0	0	11·3
100 ml. chlor-meth.19:1	0	0	13·7
100 ml. chlor-meth.12:1	0	0	12·0
100 ml. chlor-meth. 9:1	$1·5 \times 10^{-2}$	2·1	8·5
100 ml. chlor-meth. 7:1	$0·4 \times 10^{-2}$	0·6	8·4
100 ml. chlor-meth. 5:1	0	0	7·8
100 ml. chlor-meth. 3:1	$3·75 \times 10^{-2}$	5·3	4·4
100 ml. chlor-meth. 1:9	$55·5 \times 10^{-2}$	78·9	2.6
250 ml. methanol	$9·15 \times 10^{-2}$	13·0	2·9
Total	70.30×10^{-2}		
Recovery of glucose	27%		

These results show that when a fairly large amount of glucose was put on the column (about 10 times more than was used in the previous experiments), less than half was eluted from the column and, of the proportion that was eluted, most was eluted in chloroform–methanol 1:2. This suggests that in the earlier experiments, where recovery of glucose was near 100%, at least half the glucose was travelling with lipid, and that in the early fractions, particularly fraction 1, the glucose was either highly solubilised by the lipid or present as a complex.

Secondly, the lipids of the [S] series which had been refractionated with chloroform–methanol were pooled again, evaporated to dryness and taken up in 10 ml. chloroform with 1·8 mg. added glucose. This was then put on a column and fractionated. The glucose was estimated as before and counts of radioactivity remaining in the lipid from the earlier work were made, with the results shown in table .XIA.

It may be seen from these results that even less glucose was eluted than before, and that the pattern of elution is different from that of the radioactivity. This suggests that the glucose was eluted in the later fractions because of its solubility in methanol rather than because of its solubility in lipid.

The experiment was repeated using lipid fractions initially eluted with pure chloroform, in the [S] series. These early fractions were pooled, dried and taken up in 3 ml. chloroform with 2·6 mg. added glucose. The fractions were collected and glucose content and radioactivity estimated. The results are shown in table .XIB.

It may be seen that, as in the case of the middle fractions, most of the glucose was eluted in the late fractions whereas the highest concentrations of radioactivity were in the early fractions.

These three results show that glucose eluted in early fractions from silicic acid columns is present with the lipids in either a solubilised or bound form.

Procedure finally adopted for Separation of Lipids.

Following the preliminary experiments described above, a slightly different method was evolved which was used for the later experiments whose results are shown in the next chapter.

5 g. silicic acid (Malinkrodt 100 mesh) was mixed with 2·5 g. Hyflo-Supercel (Hopkins & Williams), washed four times with chloroform-methanol 2:1 on a Buchner funnel, poured into the glass column and packed down with chloroform-methanol 4:1 or 9:1 under a nitrogen pressure of 2-4 lbs./sq.in. This gave a column of length 17 cm., diameter 1 cm., dead space 5 ml. and flow-rate of eluent of about 1 ml./min.

50 mg. lipid extract was taken up in 1 ml. chloroform-methanol 4:1 or 9:1, put on the column and eluted with 50 ml. of each of the solvent mixtures chloroform-methanol 4:1, 3:2 and 1:4, or 9:1, 4:1, 3:2 and 1:4.

The eluent was collected in 5 ml. fractions. From each fraction small portions were taken for R.A. counts and for phosphorus estimations, and from the results of these an elution curve was drawn showing the peaks of phosphorus-containing material. The fractions constituting a single peak were pooled, dried for weighing, then either separated further by thin-layer chromatography or used for a rapid analysis by infra-red spectroscopy.

Estimation of Phosphorus.

The method of Taussky & Shorr (1953) was used, as modified by Rathbone (1962). 1 ml. of each fraction was dried by gentle heat in a small Pyrex tube; 0·1 ml. acid mixture 10 N H_2SO_4 : 70% perchloric acid, 1:1, was added, and the tubes placed on a sand bath at 120° for 15-24 hours. On cooling, 3 ml. developing reagent was added, mixed and allowed to stand for 10 min.; then the blue colour which developed was measured photometrically at 690 mμ on the Optica spectrophotometer.

The developing reagent was made by adding 5 g. ferrous sulphate to 10 ml. 10% ammonium in 10 N sulphuric acid, and diluting to 200 ml. with water.

A stock solution of phosphate was used containing 0·5853 g. potassium acid phosphate per litre (133·3 μg.P/ml.). 0.05, 0.10 and 0·15 ml. were used to construct a standard curve, and the phosphorus content per 5 ml. eluent was calculated from this curve.

Thin-Layer Chromatography.

The procedure followed in this laboratory by Ann C. Dawson, Judith M. Spencer and Mary L. Forsling was essentially similar to that described in the literature, for instance Randerath (1963).

Silica gel nach Stahl (Merck) was mixed with water (1:2 by weight) and spread on glass plates 20 cm. square in a layer 0·02 mm. - 0·05 mm. thick. The plates were then dried by heating and stored in a dessicator.

The lipid mixture to be separated was taken up in chloroform-methanol 4:1, in a solution of known concentration. It was applied in spots of 20 μg., or in a band up to 1 cm. wide containing up to 100 μg.,

for a layer 0·02 mm. thick. Thicker layers were capable of clearly separating greater quantities.

The lipids were separated in an air-tight tank by ascending chromatography, using the solvent mixture (developing reagent) chloroform-methanol-water, 80:30:3. For fast-moving lipids, chloroform alone was sometimes used. The length of the run was 10 cm. and it took about 20 minutes to complete.

The lipid spots were visualised by various staining techniques: iodine vapour as a general lipid stain; ammonium molybdate reagent for phosphate groups; ninhydrin reagent for amino groups; Dragendorf reagent for choline; rhodamine 6-G, followed by viewing under ultraviolet light, to distinguish between basic and acidic phosphatides. (Skidmore & Entenman, 1962; Skipski, Peterson & Barclay, 1962.)

The following pure lipids were used as markers: cardiolipin, cerebrosides, synthetic cephalin, phosphatidylcholine, phosphatidyl-inositol and phosphatidylserine. Using the two methods - identification of constituent groups and comparison of Rf values with known compounds - it was possible to identify most of the lipid spots separated from red cell and placental lipids.

If the applied lipid contained radioactivity, the chromatogram was scanned by placing it on a trolley moving under a thin-window Geiger counter. It was found that free glucose, applied alone, was not eluted by the rising solvent but remained at the origin. Lipids mixed with glucose before extraction,however, showed radioactivity in the lipid spots.

Infra-red Spectroscopy.

Potentially, the infra-red spectrophotometer is an ideal instrument for detecting small amounts of a substance within a mixture, and for gaining some knowledge of its structure by assigning the absorption bands to certain molecular groupings. Schwarz (1960) has described how tissue lipids in a cell extract may be identified by matching with a library of absorption curves of pure, known compounds. It was hoped that it might prove possible to locate a lipid-glucose complex in this way, without having to resort to chemical means which necessarily destroy the hypothetical complex.

It was not found possible to locate the carrier substance or complex in the short time available, but nevertheless the technique used and some of the findings will be briefly described.

The instrument used was a Perkin-Elmer model 337 grating spectrophotometer, double-beam, scanning the range 4000 $cm.^{-1}$ - 400 $cm.^{-1}$ (2·5μ - 25μ wavelength). Cells with windows of potassium bromide were used. The most satisfactory solvents were found to be carbon tetrachloride in the range 2·5μ - 7·5μ, and carbon disulphide in the range 7·5μ - 25μ. Substances which were not easily soluble in these solvents were studied in the mulled state: the substance was mixed thoroughly with liquid paraffin (or "Nujol"), then placed between two KBr plates. An insoluble substance could also be ground to a fine powder with KBr crystals and compressed in a hydraulic press into a clear disc.

Pure phospholipids, triglycerides (olive oil), oleic acid, cholesterol, sugars and inhibitors were used to build up a library of reference spectra with which unknown substances or mixtures could be

compared. It was found that absorptions at certain wavelengths could be correlated with certain groups, and that cholesterol could be estimated fairly quantitatively from its absorption at 3·2μ (2950 $cm.^{-1}$).

To give a clearly distinguishable spectrum, it was necessary to use 5 mg. material. In the present work, fractions from a column on which 50 mg. red cell lipids had been separated were used, which meant that, to be clearly identified, a particular solution should constitute at least 10% of the total lipid mixture. The carrier substance would not be present at anywhere near this proportion.

EXPERIMENTAL SECTION II

Phospholipids from Cell Membranes

CHAPTER 5

Results obtained for sheep placental phospholipids

Column Chromatography.

The column chromatographic technique finally adopted, and described in the previous chapter, was used first to separate the lipids from sheep placental tissue. Radioactivity was present in the extracted lipids as the result of the injection of [^{14}C] glucose or [^{14}C] fructose into the umbilical vein three hours before separation of the foetus from the placenta.

Six sheep were used:-

Sheep	Foetal Age	Sugar Injected	Method of Extraction
I	104 days	1 m.curie [^{14}C] fructose	Described in previous chapter
II	104 days	3 m.curie [^{14}C] fructose	Extracted with chlor-meth. 2:1
III	109 days	3 m.curie [^{14}C] glucose	" " " " "
IV	136 days	3 m.curie [^{14}C] glucose	" " " " "
V	134 days	3 m.curie [^{14}C] fructose	" " " " "
VI	147 days (term)	No sugar injected	" " " " "

For each placenta II-V the chloroform-methanol extract was finally evaporated to dryness in two portions: the "acetone-soluble" and the "acetone-insoluble" portions. Each contained the same lipids, although in different proportions, but the acetone-insoluble precipitate was in a purer form, being free from water and acetone-soluble contaminants such as free sugars.

The dry weights recovered were variable: 200-300 g. placental tissue yielded 1-5 g. lipid. The amount of acetone-insoluble material precipitated depended on the conditions such as proportion of acetone added, temperature, state of aggregation of the lipid molecules; the

weight of acetone-soluble material obtained probably depended largely on the efficiency of the drying process, since lipids, and particularly phospholipids, tend to form stable aggregates with water.

Recoveries of radioactivity, shown below, were calculated from counts measured on samples of the lipids before fractionation. The considerable radioactivity remaining in the aqueous parts of the extracts was difficult to measure accurately and has been ignored. A sample of the [^{14}C] fructose injection was found to give 681 x 10^6 counts/min./m.curie. From this, the total radioactivity in the lipids as a percentage of the original injection was calculated.

Sheep	R.A. recovered in lipid	Lipid R.A. as % of injection
II	90,000 counts/min.	0·004%
III	520,000 counts/min.	0·025%
IV	777,000 counts/min.	0·038%
V	123,000 counts/min.	0·006%

It may be seen that in the glucose-injected sheep (III & IV) about six times as much radioactivity was recovered as in the fructose-injected sheep (II & V). The higher level of [^{14}C] in placental lipids after glucose injection could be due to more glucose passing back across the placental barrier to the maternal circulation, or to a more rapid metabolic incorporation of glucose than fructose into placental lipids.

The dry lipids obtained were applied to silicic acid columns in 50 mg. portions, as described in the previous chapter, eluted with chloroform-methanol mixtures and the phosphorus content and radioactivity of each 5 ml. fraction estimated. The results were plotted as elution curves, and the curves for placentae IV and V are shown in figs. 6 and 7.

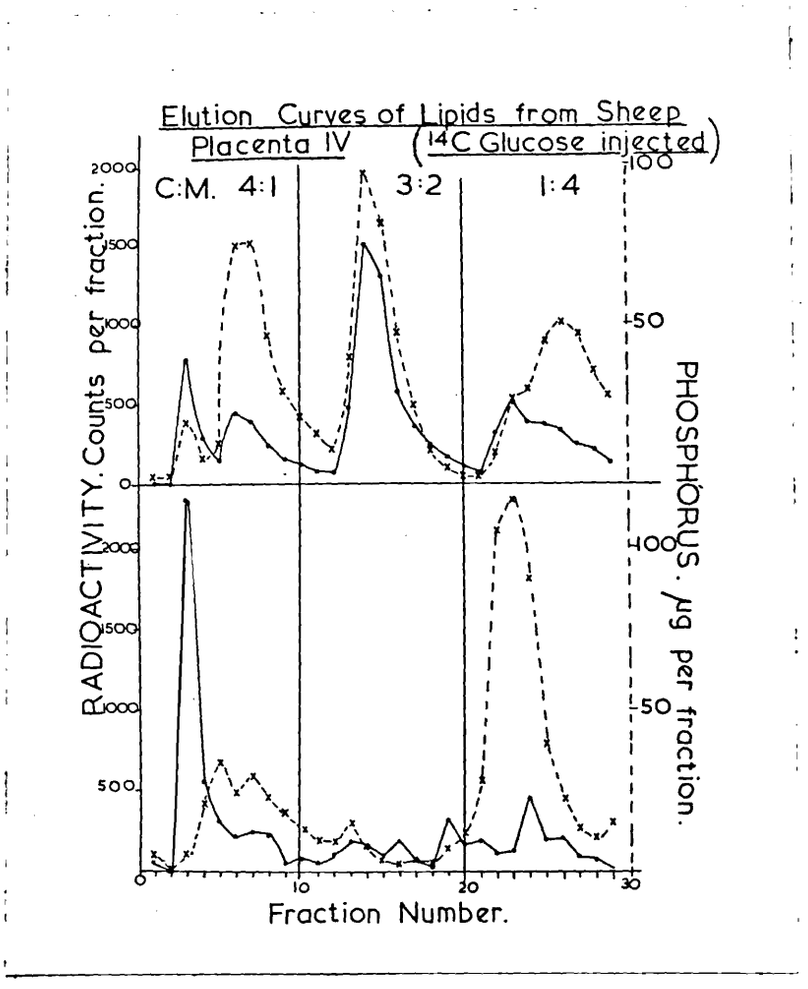


Fig. 12. Elution curves of sheep placental phospholipids from placenta IV (^{14}C-glucose injected), obtained by separation on silicic acid columns.

(a) Acetone insoluble, 50 mg.

(b) Acetone soluble, 50 mg.

Continuous line: Radioactivity, counts per fraction.

Broken line: Phosphorus, µg, per fraction.

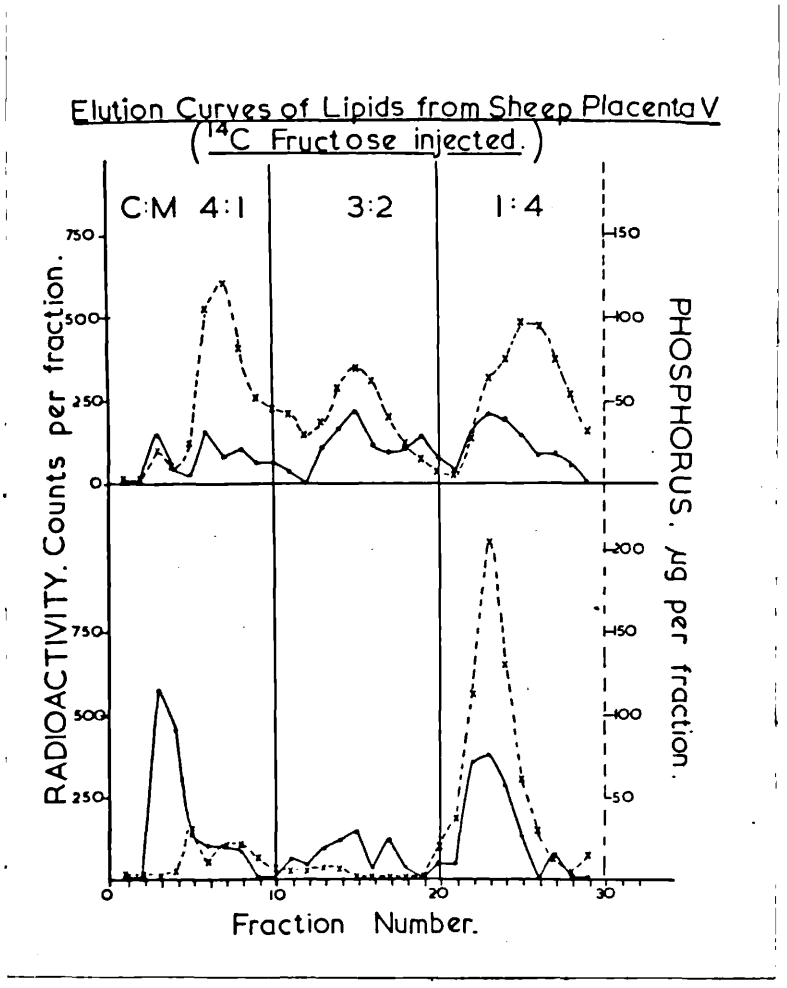


Fig. 13. Elution curves of sheep placental phospholipids from placenta V (^{14}C–fructose injected), obtained by separation on a silicic acid column.

(a) Acetone insoluble, 50 mg.

(b) Acetone soluble, 50 mg.

Continuous line: Radioactivity, counts per fraction.

Broken line: Phosphorus, µg. per fraction.

Fig. 12 shows the elution pattern of phosphorus and radioactivity in the placental lipids of the [^{14}C]-glucose-injected sheep. The acetone-soluble and acetone-insoluble portions contained a total of 6,200 and 9,900 counts/min./50 mg. respectively.

It may be seen that the acetone-insoluble lipids show five phosphorus elution peaks: A and B eluted with chloroform-methanol 4:1, C with 3:2, and D and E with 1:4. Radioactivity is associated with the phosphorus except in the last peak. The acetone-soluble lipids are different in that peaks B, C and E are greatly reduced, while peak D is larger. In both portions the greatest radioactivity:phosphorus ratio was found in peak A.

Fig. 13 shows the elution pattern of phosphorus and radioactivity in the placental lipids of the [^{14}C]-fructose-injected sheep. The acetone-soluble and acetone-insoluble portions contain a total of 3,400 and 2,500 counts/min./50 mg. respectively. Although there is less radioactivity than in the previous figure, it nevertheless follows the phosphorus elution curve in a similar way, with again a high R.A.: P in the early fractions of the acetone-soluble lipids.

If the first elution solvent mixture used was chloroform-methanol 9:1, then peak A was eluted in 9:1 and peak B in 4:1, whether acetone-soluble or acetone-insoluble.

Recoveries of both phosphorus and radioactivity was usually close to 100% and varied between 90% and 105%.

TABLE XII

Elution of free [^{14}C]-glucose and [^{14}C] fructose from silicic acid columns.

Elution Solvent	[^{14}C] Glucose		[^{14}C] Fructose	
Chlor-Meth.	R.A. cnts./min.	% total	R.A. cnts./min.	% total
9:1	45	0.6	134	0.8
4:1	142	1.9	272	1.7
3:2	6,989	95.2	14,450	91.2
1:4	167	2.3	981	6.2

TABLE XIII

Sheep placental phospholipids: recoveries of phosphorus and radioactivity in elution peaks from column chromatography.

A. ^{14}C Glucose injected.

	Placenta III				Placenta IV			
	Acetone Sol.		Acetone Insol.		Acetone Sol.		Acetone Insol.	
Peak	P%	R.A.%	P%	R.A.%	P%	R.A.%	P%	R.A.%
A	-	-	3·4	3·3	12·6	51·1	3.6	10·8
B	31·2	30·5	19·4	12·2	13·8	8·9	30·9	16.1
C	3·8	12.2	46·5	49·2	5·1	11·2	35.4	47·7
D	65·0	57·3	30·7	35·3	68·4	28·8	30·1	25·3

B. ^{14}C Fructose injected.

	Placenta II				Placenta V			
	Acetone Sol.		Acetone Insol.		Acetone Sol.		Acetone Insol.	
Peak	P%	R.A.%	P%	R.A.%	P%	R.A.%	P%	R.A.%
A	-	-	24·7	19·3	8·4	40·0	4·3	18.0
B	-	-	7.4	6.0	12.9	6·1	41·8	17·4
C	-	-	46·2	47·0	1·8	16·2	21·5	33·7
D	-	-	21.7	28·1	76·9	37·8	32·4	30·9

In an experiment performed to determine where free glucose or fructose would be eluted using the present solvent mixtures, if applied to the column alone or in the presence of lipid, it was found that in either case the results obtained were as shown in table XII.. From these results it was deduced that radioactivity eluted from the lipid extracts with chloroform–methanol 3:2 may indicate the presence of free glucose, particularly in the acetone–soluble portions where some free glucose would be expected, but that the high radioactivity obtained in the early fractions cannot be due to free glucose but must indicate the presence of either glucose in a solubilised form or lipid–soluble material into which [^{14}C] has become incorporated by metabolism.

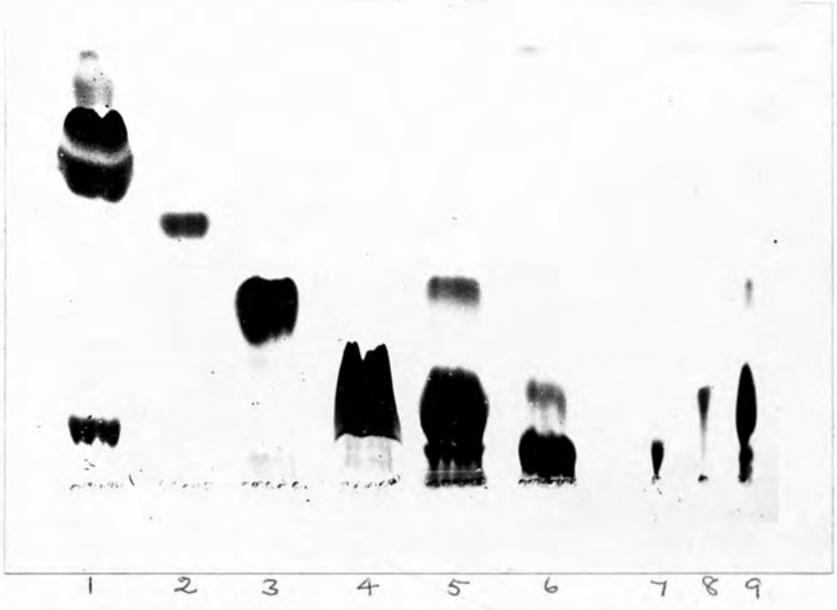
The results obtained for the fractions comprising the individual peaks were pooled, and their phosphorus and radioactivity contents expressed as a percentage of the total. The results obtained were as shown in table XIII.. Peaks D and C, being sometimes difficult to separate, have been pooled together.

It may be seen that the results for placentae II and III are fairly similar to those for IV and V already discussed. However, nothing may be concluded from them until an attempt has been made to identify the lipids eluted in each peak.

Identification of Lipids.

By the thin–layer chromatographic technique described previously, and by matching the unknown components against the known reference substances which were then available (lecithin prepared from egg–yolk, sphingomyelin and a cyclic acetal phospholipid or plasmalogen), many of the components of the peaks were identified.

(i)



(ii)

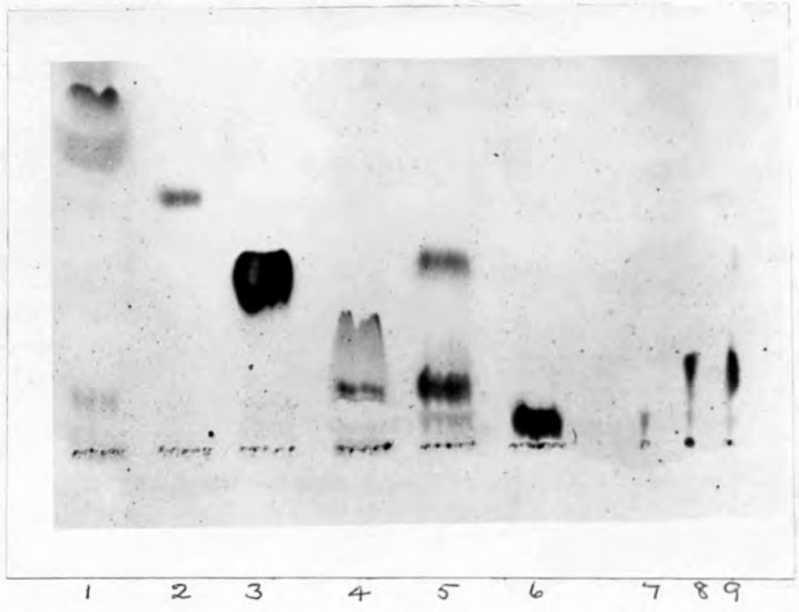
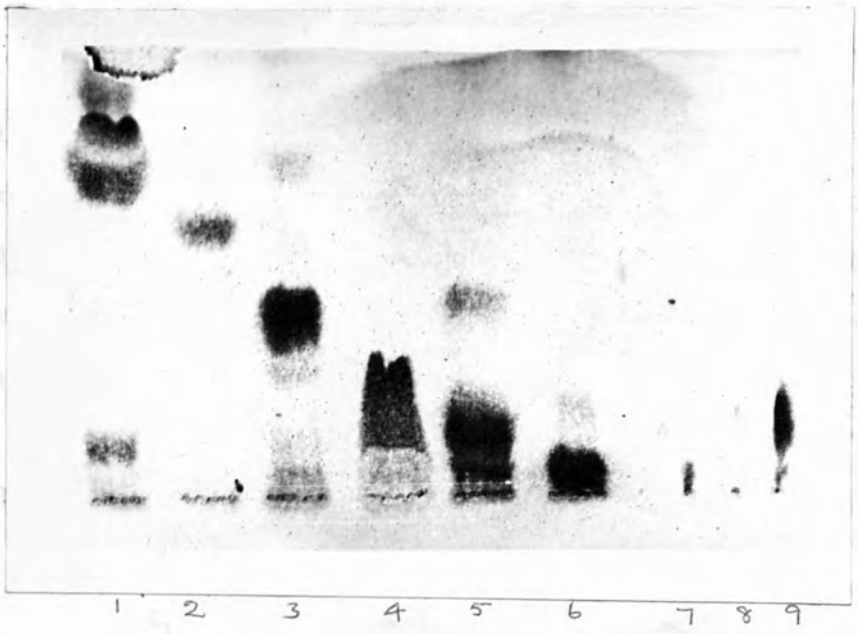


Fig. 14. Thin-layer chromatograms of acetone-insoluble lipids from sheep placenta IV, (i) treated with iodine vapour (general lipid stain), (ii) treated with ninhydrin reagent (stain for amino groups).
Lipid eluted from the silicic acid column, as shown in fig. 6, was applied to the plate as follows: 1. Peak A. 2. Early part of peak B. 3. Later part of peak B. 4. Peak C. 5. Peak D. 6. Peak E. 7. Sphingomyelin marker. 8. Cyclic acetal phospholipid marker. 9. Egg yolk extract marker for lecithin.

(i)



(ii)

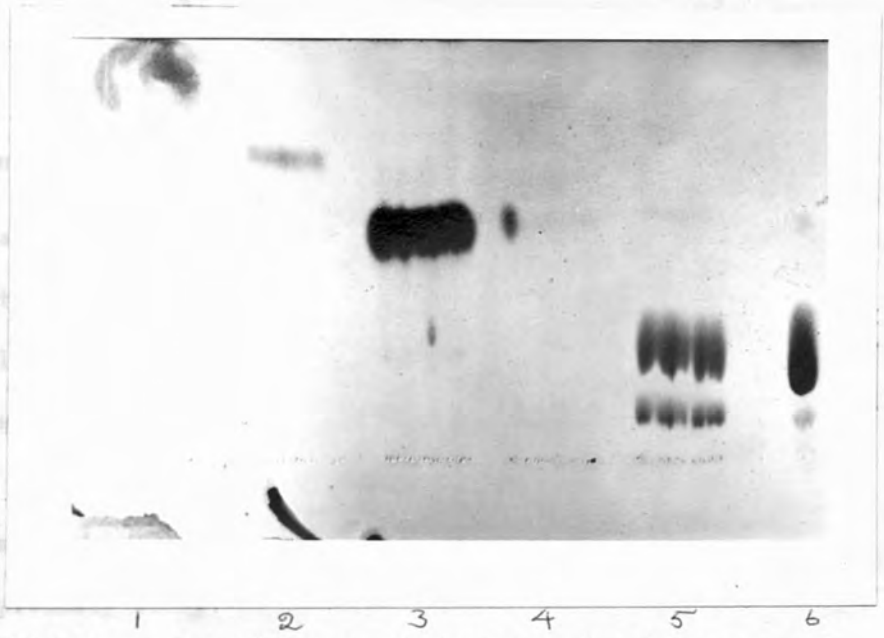


Fig. 15. Thin-layer chromatograms, stained for phosphate groups: (i) sheep placenta IV, acetone-insoluble lipids; (ii) sheep placenta III, acetone-soluble lipids. (i) numbered as in fig. 14; (ii) applied to the plate as follows: 1. Peak A. 2. Peak B. 3. Peak C. 4. Peak D. 5. Peak E. 6. Egg yolk extract.

In addition, the peaks from one column were tested for the presence of phosphoinositides: the pooled fractions were dried, weighed and hydrolysed to split off free inositol. The inositol was then estimated by Dr. D.A. Nixon, St. Mary's Hospital Medical School, London, by a microbiological technique. The results are shown below:-

	Peak	Dry Wt. Lipid	Inositol
Acetone-insoluble	A	4·8 mg.	0·75 μg.
	B	11·8	2·6
	C_1	8·5	49·5
	C_2	11·5	505·0
	D	9·1	12·2
Acetone-soluble	C	0·4	1·6

It may be concluded from these results that phosphoinositides are found almost exclusively in peak C of the acetone-insoluble lipid.

The lipid fractions were pooled and applied to the thin-layer plates, developed, then stained, as shown in figs. 14 & 15.

Peak A contained the neutral lipids and sterols, and also two or three fast-moving phosphorus-containing components, ninhydrin negative, which were probably polyglycerophosphatides or phosphorus-containing degradation products.

Peak B contained the cephalins: phosphatidylserine travelled faster and was found in the first half of the peak; phosphatidylethanolamine, which was present in large amounts, was found in the second half. Both gave positive reactions for phosphorus and amino-groups, but it was possible to distinguish between them by their relative mobilities and by

staining with rhodamine 6-G and viewing under ultra-violet light, when one appears pink and the other yellow.

Peak C contained phosphoinositide and also small, varying amounts of ninhydrin-staining material which was probably lecithin. Although ninhydrin is supposed to stain only amino-groups, it may be seen from fig. 13 that the choline group of lecithin is stained as well.

Peak D contained most of the lecithin and probably the lyso-phosphatides.

Peak E contained all the sphingomyelin, with a little lecithin.

The acetone-soluble lipids contained a higher proportion of neutral lipids. The dry weights of the fractions showed that about half the weight of the lipid was eluted in the early fractions, in peak A, and was shown by infra-red spectroscopy to be mainly sterols and glycerides. Thin-layer chromatography showed that there were also some fast-moving phosphorus-containing substances, and some phosphatidylserine.

Peak B contained phosphatidylethanolamine.

Peak C contained too little lipid to be detected on the thin-layer chromatogram.

Peak D contained lecithin and lysophosphatides, and possibly some plasmalogens.

Peak E was small, and contained only a little sphingomyelin.

Distribution of Radioactivity.

Free [^{14}C]-glucose or [^{14}C]-fructose was applied to a thin-layer plate, developed as described for the lipid extracts, then scanned by moving the chromatogram under a Geiger counter. The radioactivity was found always to remain at or near to the origin.

TABLE XIV

Uptake of [^{14}C] hexose by placental lipids in a short space of time.

	Placenta VI			
	1	2	3	4
	[^{14}C] Fructose		[^{14}C] Glucose	
Radioactivity	20 secs.	60 secs.	20 secs.	60 secs.
R.A. added	800,000 c/m	84,000 c/m	1,380,000 c/m	1,380,000 c/m
R.A. recovered	3,980 c/m	450 c/m	2,430 c/m	3,830 c/m
% recovery	0·49%	0·52%	0·18%	0·28%
Dry Weights				
Acetone-soluble	290 mg.	230 mg.	223 mg.	244 mg.
Acetone-insoluble	140 mg.	120 mg.	57 mg.	162 mg.
R.A. Counts/50 mg.				
Acetone-soluble	625	54	440	267
Acetone-insoluble	132	82	396	785

The radioactivity in the lipid extracts was found always to move with the lipid spots, and never to remain at the origin. In peak A, the radioactivity was greatest in the Rf range 0·7 - 1·0, coinciding with the phosphorus-positive, ninhydrin-negative spots. These peaks were more marked in the [^{14}C]-glucose-injected sheep than in the [^{14}C]-fructose-injected sheep. In the other peaks the radioactivity moved with the main phospholipid spot.

These lipids had been in contact with [^{14}C] hexose for three hours, and it is not known what changes the [^{14}C] had undergone in that time.

To investigate the uptake of hexose by placental lipids in a short space of time, placenta VI was used, from the sheep which had no [^{14}C] hexose injection.

Uptake of [^{14}C] hexose by placental lipids in a short space of time.

The cotyledons from placenta VI were divided into four groups of approximately 20 g. and stored in the deep freeze until required. To two groups [^{14}C] glucose was added, and to the other two groups [^{14}C] fructose. In each case 20 g. placental tissue was macerated with 40 ml. 0·9% saline for 45 seconds to give a homogenate. 2·5 ml. of a standard solution of [^{14}C] glucose or [^{14}C] fructose, containing a known amount of radioactivity, was added to the homogenate and mixed for either 20 or 60 seconds, then immediately poured into 210 ml. chloroform-methanol 2:1, mixed vigorously for 30 seconds, then allowed to stand overnight. The extract was treated similarly to the previous lipid extracts, and divided into acetone-soluble and acetone-insoluble portions.

TABLE XV

Elution of lipids from Sheep Placenta VI after addition of [^{14}C] Hexose.

A. [^{14}C] Fructose added.

Peak	20 secs.				60 secs.			
	Acetone Sol.		Acetone Insol.		Acetone Sol.		Acetone Insol.	
	P%	R.A.%	P%	R.A.%	P%	R.A.%	P%	R.A.%
A	0·8	2·7	3·6	6·1	11·2	15·0	2·9	22·6
B	33·4	9·6	30·3	39·8	24·7	0	27·3	32·3
C	5·3	68·7	42·5	44·6	2·9	84·6	19·0	29·0
D	56·1	19·0	23·6	9·5	57·8	0	48·9	16·1

B. [^{14}C] Glucose added.

Peak	20 secs.				60 secs.			
	Acetone Sol.		Acetone Insol.		Acetone Sol.		Acetone Insol.	
	P%	R.A.%	P%	R.A.%	P%	R.A.%	P%	R.A.%
A	19·6	17·2	6·8	8·5	19·2	0	4·5	2·7
B	30·3	13·3	23·8	11·2	17·9	36·5	20·1	10·3
C	3·5	56·2	31·9	80·3	1·6	49·4	18·4	73·8
D	40·9	13·3	31·7	0	55·8	0	55·1	9·1

The recoveries of phosphorus and radioactivity are shown in table XIV. It may be seen that the amount of hexose taken up in the lipids is not great. Nevertheless, more than half the total radioactivity recovered was present in the acetone-insoluble lipids after mixing for 60 seconds, and this is unlikely to be due to free glucose. Since the mixings and extractions were performed at room temperature it is unlikely that much hexose had been incorporated metabolically.

The lipids were eluted on silicic acid columns as before, and the results are shown in table XV. The peaks of phosphorus and radioactivity were subsequently separated by thin-layer chromatography, and the chromatograms scanned for radioactivity. The only peaks with high enough activity to give clear results were peaks C, i.e. those eluted with chloroform-methanol 3:2, and in these the radioactivity remained at the origin, suggesting that the hexose was present in the free state. In the case of the acetone-insoluble lipids it seems unlikely that hexose was in the free state in the original extract; it was probably either solubilised in the lipid or formed a lipid-glucose complex which subsequently became dissociated during column chromatography. The levels of radioactivity in the other peaks is higher than could be accounted for by the elution pattern of free hexose, but could be due to solubilisation in lipid alone.

That concludes the work done on sheep placental tissue. From the results obtained it cannot be decisively concluded that a hexose-lipid complex had been extracted which could be involved in hexose transfer in the placenta, but nevertheless the results do show the possibility of the existence of such a complex.

This survey was made primarily because of the high levels of [^{14}C] sugars being used by Professor Huggett and his co-workers in these experiments. Also, although the red cell has proved most convenient for kinetic studies, it is not necessarily the tissue with the glucose transfer system in the greatest amount or most readily extractable form. The results obtained did not suggest any advantages of placental tissue as a site for studying this problem, but they did emphasize the importance of adequate radioactive labelling. This point, and the experience of handling lipid fractions labelled with [^{14}C] tracer, proved valuable for the work on the central problem with red cells described in the next chapter.

EXPERIMENTAL SECTION II

Phospholipids from Cell Membranes

CHAPTER 6

Results obtained for Human and Sheep Red Cell Lipids

TABLE XVI

Uptake of [^{14}C] Glucose by Human Red Cell Lipids in (i) 20 seconds, (ii) 80 seconds.

A. Recoveries of Dry Weights and Radioactivity.

	20 seconds			80 seconds		
	Ac.Sol.	Ac.Insol.	Total	Ac.Sol.	Ac.Insol.	Total
Dry Weight (mg.)	83	11	94	98	5	103
R.A. added (c/m)			1,378,000			1,378,000
R.A. recovered (c/m)	14,000	440	14,440	4,700	190	4,890
% recovery			1·05%			0.36%

B. Radioactivity and Phosphorus contents of elution peaks.

Peak	20 seconds				80 seconds			
	Acetone Sol.		Acetone Insol.		Acetone Sol.		Acetone Insol.	
	P%	R.A.%	P%	R.A.%	P%	R.A.%	P%	R.A.%
A	1·9	0.8	18·2	24·7	6·5	1·0	42·7	7·7
B	30·8	1.2	18·3	22·4	38·3	1·0	20·7	7·5
C	2·4	96.3	31·4	48.9	5·0	95.0	18·0	18.8
D	60·7	1.7	32·0	4.0	45·1	0	18·0	66.1

Distribution of Radioactivity in Red Cell Lipids.

Using the same technique as for placental homogenates to measure the uptake of [^{14}C] hexose in a short space of time, red cell lipids were found to have a rather different radioactivity distribution.

100 ml. packed, washed human red cells were mixed for either 20 or 80 seconds with 2 ml. [^{14}C] glucose solution, then poured into 420 ml. chloroform–methanol 2:1, mixed thoroughly and left to stand. The extract was obtained, as before, in acetone–soluble and acetone–insoluble portions. Recoveries of dry weight and radioactivity, and also the result of elution of the lipids on silicic acid columns, is shown in table XVI. The bulk of the radioactivity added to the red cells remained in the aqueous phase and in the solid residue.

It may be seen that most of the radioactivity in the lipids was found in the acetone–soluble portions, in peak C; thin–layer chromatography showed this to be free glucose.

Experiments have already been described in Chapter 5 which showed that, for red cells, better recovery of radioactivity was obtained by extracting with n–butanol than with chloroform–methanol 2:1. These results will now be discussed in more detail.

The radioactivity recovered, per 50 mg. extract, was as follows:–

Human red cells,	chloroform methanol	15,500 counts/min./50 mg.
" " "	n–butanol	26,300 counts/min./50 mg.
Sheep red cells,	chloroform–methanol	5,470 counts/min./50 mg.
" " "	n–butanol	17,000 counts/min./50 mg.

In each case a large proportion of the radioactivity remained in the solid residue. None of the extracts was divided into acetone–soluble and acetone–insoluble portions.

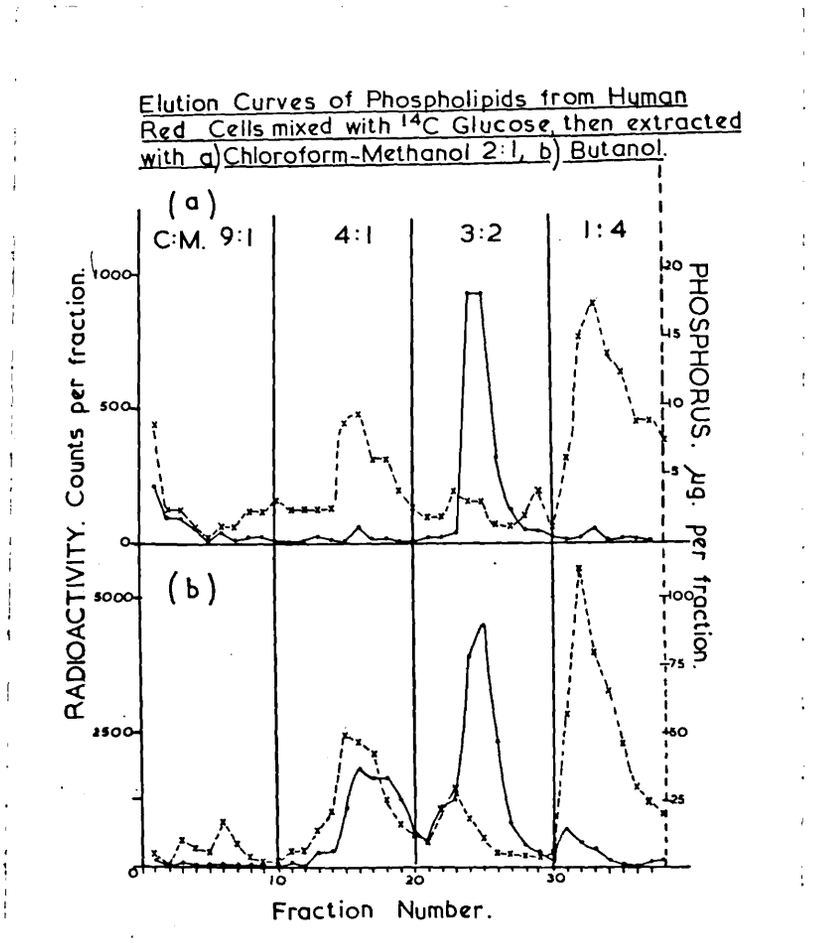


Fig. 16. Elution curves of human red cell phospholipid. The red cells were mixed with [^{14}C] glucose for 20 seconds, then extracted with (a) chloroform-methanol 2:1 (approximately 10 mg.), (b) n-butanol (50 mg.).

Continuous line: Radioactivity, counts per fraction.

Broken line: Phosphorus, µg. per fraction.

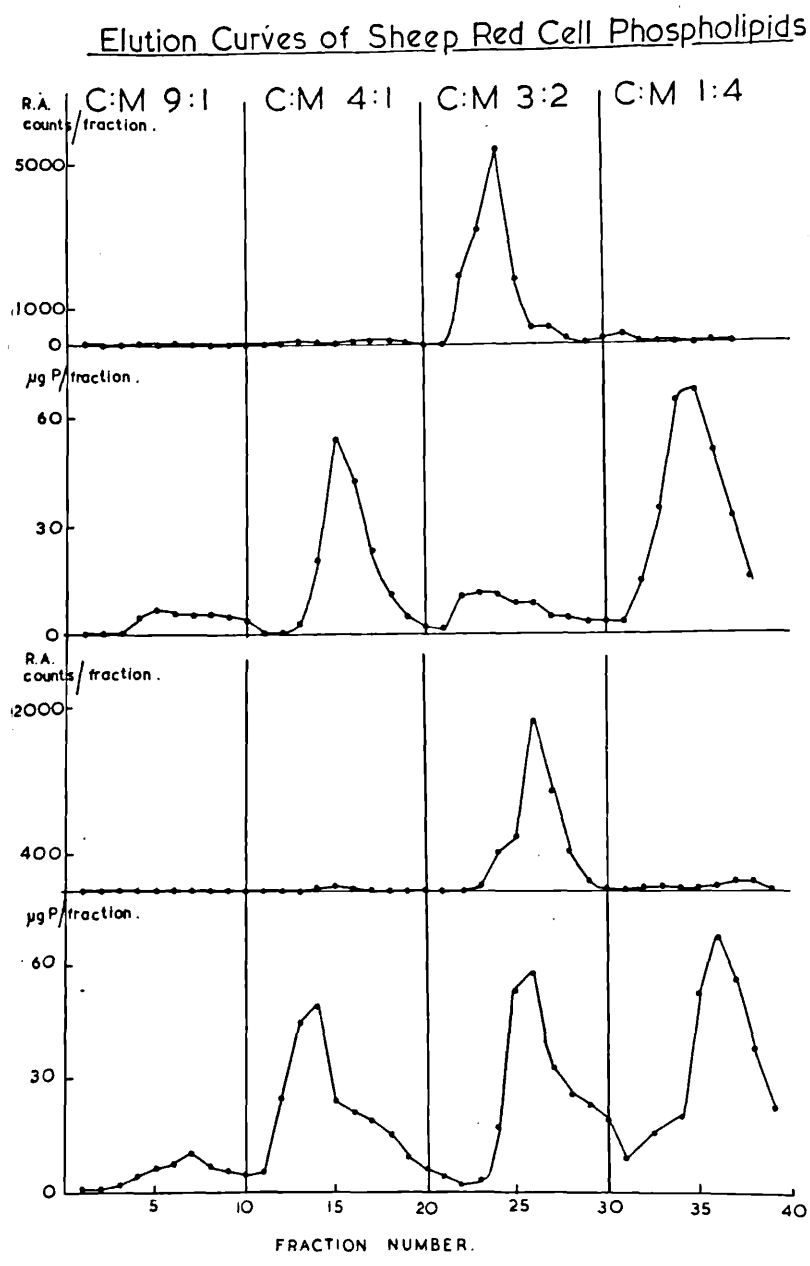


Fig. 17. Elution curves of sheep red cell phospholipids. The red cells were mixed with [^{14}C] glucose for 20 seconds, then extracted with (a) chloroform–methanol 2:1, (b) n-butanol.

It was to be expected that the chloroform–methanol extracts should contain less [^{14}C] glucose than the butanol extracts since, after dividing the wet chloroform–methanol extract into two phases by spinning, the aqueous phase, which contained about 40% of the radioactive counts, was put on one side. The entire butanol extract, on the other hand, was taken to dryness without separation of the aqueous phase. It might have been expected from this that the butanol extracts would contain a higher percentage of free glucose. This was not found to be the case in the human red cell extracts, although it was in the sheep.

Elution of the extracts on silicic acid columns gave the results shown in figs. 16 and 17.

The phosphorus elution curves showed several intersting features. Peak A was smaller in the sheep than in the human red cell extract, so presumably contains less of the fast–moving phosphorus–containing lipids. Peak B was similar in size, containing similar amounts of phosphatidyl-ethanolamine and phosphatidylserine. Peak C was reduced in the human extracts and in the sheep butanol extracts, as compared with the placental extracts, presumably due to poor extraction of phosphoinositide or to a lower content in the original tissue. The early part of peak D was almost absent in the sheep extracts, as would be expected from the known low lecithin content of sheep red cells already mentioned in the Introduction. The sheep extracts, however, contained more sphingomyelin, eluted in the later part of chloroform–methanol 1:4.

The elution pattern of radioactivity shows higher [^{14}C] glucose in peaks A and B in the human, particularly in the butanol extract. The

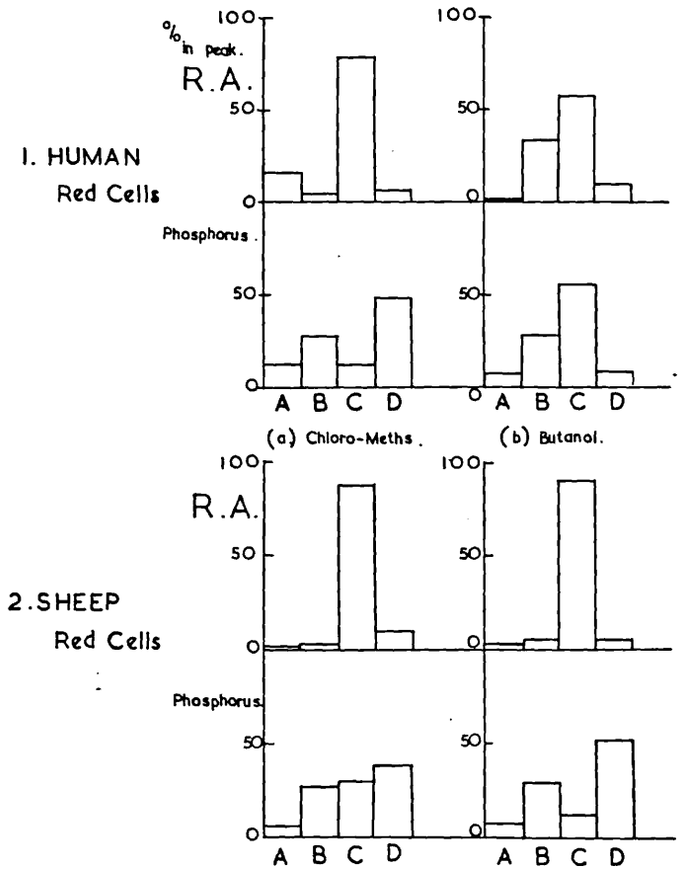


Fig. 18. Percentage recoveries of phosphorus and radioactivity in the elution peaks of (1) human and (2) sheep red cells, extracted with (a) chloroform–methanol 2:1 and (b) n-butanol.

radioactivity and phosphorus in the peaks, expressed as percentages, is shown in fig. 18. The lecithin and sphingomyelin parts of peak D have been pooled.

It may be seen from these results that the elution of phosphorus is fairly consistent, except in the sheep red cell chloroform–methanol extract where some of the material from peak D appears to have been eluted in peak C (possibly as lyso-phosphatides). This consistency makes the higher radioactivity in the early peaks of the human extracts the more striking. The chloroform–methanol extract would be expected to contain a higher proportion of neutral lipids than the butanol extract: these would be eluted in peak A, carrying with them other lipid and non-lipid substances in solubilised form, including [^{14}C] glucose. In the butanol extract, the fast-moving phosphorus-containing lipids move more slowly and appear to hold back the [^{14}C] glucose with them. It is also probable that butanol, being a more powerful extraction solvent than chloroform–methanol, has released more phospholipids from the tissue, for instance from the proteo-lipids, and that these have a high affinity for glucose.

It was tempting at this stage to correlate certain lipids in the human red cell extracts, particularly the faster-moving phospholipids such as cardiolipin, phosphatidylserine and phosphatidylethanolamine, with their glucose affinities, and to point to them as possible membrane carriers. However, the evidence obtained using the methods described was not conclusive. For the results to be convincing, the amount of [^{14}C] glucose recovered in the form of a carrier complex should be predictable, but it could not be predicted for two reasons. First, it was not known how many

carrier components there are in the red cell membrane, since the rate of glucose transfer measured could be maintained by a large number of components operating slowly or a small number operating rapidly. Secondly, the carrier-glucose complex must be reversible, and the proportion of the complexes which would withstand the extraction and separation procedures was unknown. Thus it was not even known whether the amounts of [^{14}C] glucose which would be extracted with the lipid carrier component from 50 ml. red cells would be detectable by the techniques used.

The next series of experiments was performed in an attempt to label the carrier components more permanently, and so to give some idea of the number of components present. For this purpose, dinitrofluorobenzene (DNFB) was chosen, which is known to react irreversibly with parts of the red cell membrane concerned with glucose transfer.

Uptake of [^{14}C] DNFB by Human Red Cells.

1-fluoro-2,4-dinitrobenzene was originally used by Sanger (1945) as an end-group reagent in protein analysis, and is known to react specifically with -SH and -NH_2 groups and tyrosine to give dinitrophenolic derivatives. Bowyer (1954) found that it inhibited glucose transfer in human red cells, both inward and outward, and that its effect was due to the action of DNFB on -SH or -NH_2 groups. Later work (Bowyer & Widdas, 1958; Sen & Widdas, 1962b) showed the inhibitory action to be irreversible, non-competitive, more rapid in the presence of glucose, and more effective for glucose exits than entries.

Although DNFB is primarily a protein reagent, and probably reacts with several of the proteins in the red cell membrane, it seemed likely that it would react with the amino-groups of the phospholipids as well.

The experiments on uptake of [^{14}C] DNFB and the simultaneous inhibition of glucose transfer were done in conjunction with Professor W.F. Widdas and Miss Mary L. Forsling, and have been briefly reported (Forsling, Remfry & Widdas, 1964).

Materials.

Red Cells. Freshly withdrawn human blood was used for experiments 1, 2 and 3; time-expired human blood for experiment 4. Red cells were washed three times, and a sample used to check that the glucose transfer mechanism was intact by the photo-electric method (Widdas, 1953).

Glucose. D-glucose (non-radioactive).

DNFB. [^{14}C] Universal labelling, 2·1 mg. (50 μcuries), obtained from the Radiochemical Centre, Amersham, Bucks. 2·1 mg. [^{14}C]-DNFB was added to 96 mg. unlabelled DNFB and made up to 19·8 ml. with absolute ethanol. For experiments 1, 2 and 3, 0.66 ml. was diluted to 6·3 ml. with 0·9% NaCl. For experiment 4, the undiluted alcoholic solution was used.

It had been found previously (Bowyer & Widdas, 1958) that a low concentration of DNFB, of the order of 1 mM., gave almost complete inhibition after several hours incubation with the red cells with little risk of haemolysis. Rubber-bulb-operated pipettes were used instead of mouth-pipettes throughout these experiments.

Method.

Four experiments were performed:-

(1) 5 ml. red cells + 1·4 mM. DNFB, incubated at 21°.

(2) 5 ml. red cells + 1·4 mM. DNFB, incubated at 28°.

(3) 5 ml. red cells + 1·4 mM. DNFB, incubated at 33°.

(4) 50 ml. red cells + 1·4 mM. DNFB, incubated at 33°.

In experiment 1 the procedure was as follows: the red cells were first incubated with approximately 76 mM. glucose at 37°, then 5 ml. red cell suspension was added to 5 ml. diluted DNFB solution at 21°, giving a final concentration of 1·4 mM. At measured time intervals samples of the inhibition-incubation mixture were taken to measure (a) inhibition of glucose transfer on the photoelectric apparatus, (b) uptake of radioactivity by the red cells.

For (b) a 1 ml. sample was added to 9 ml. ice-cold buffered saline, mixed well, divided into two portions and each portion spun down. 0·2 ml. supernatant was taken from each for radioactive counting. The rest of the supernatant was discarded. The cells were washed twice more, taking 2 x 0·2 ml. samples of the supernatant of the second washing. Finally the red cells were suspended in 1 ml. saline, giving a total volume of about 1·2 ml., and 2 x 0·2 ml. samples of the suspension were taken for counting and 2 x 0·2 ml. for haemoglobin estimations.

To check the irreversibility of the DNP derivative formed in the membrane, a 1 ml. sample was removed after 5 minutes incubation, added to 18 ml. ice-cold, glucose-containing buffered saline, mixed, spun, washed again in saline at room temperature, then the red cells resuspended in 2 ml. DNFB-free supernatant and incubated for about 2 hr. 40 min. at 21°. At the end of the incubation period, 1 ml. of the suspension was added to 9 ml. ice-cold buffer, spun, and 2 x 0·2 ml. of the supernatant taken for counting. The cells were then resuspended in 1 ml. buffer, 2 x 0·2 ml. samples taken for counting and 2 x 0·2 ml. for haemoglobin estimations. These samples were differentiated from the other series by the prefix X.

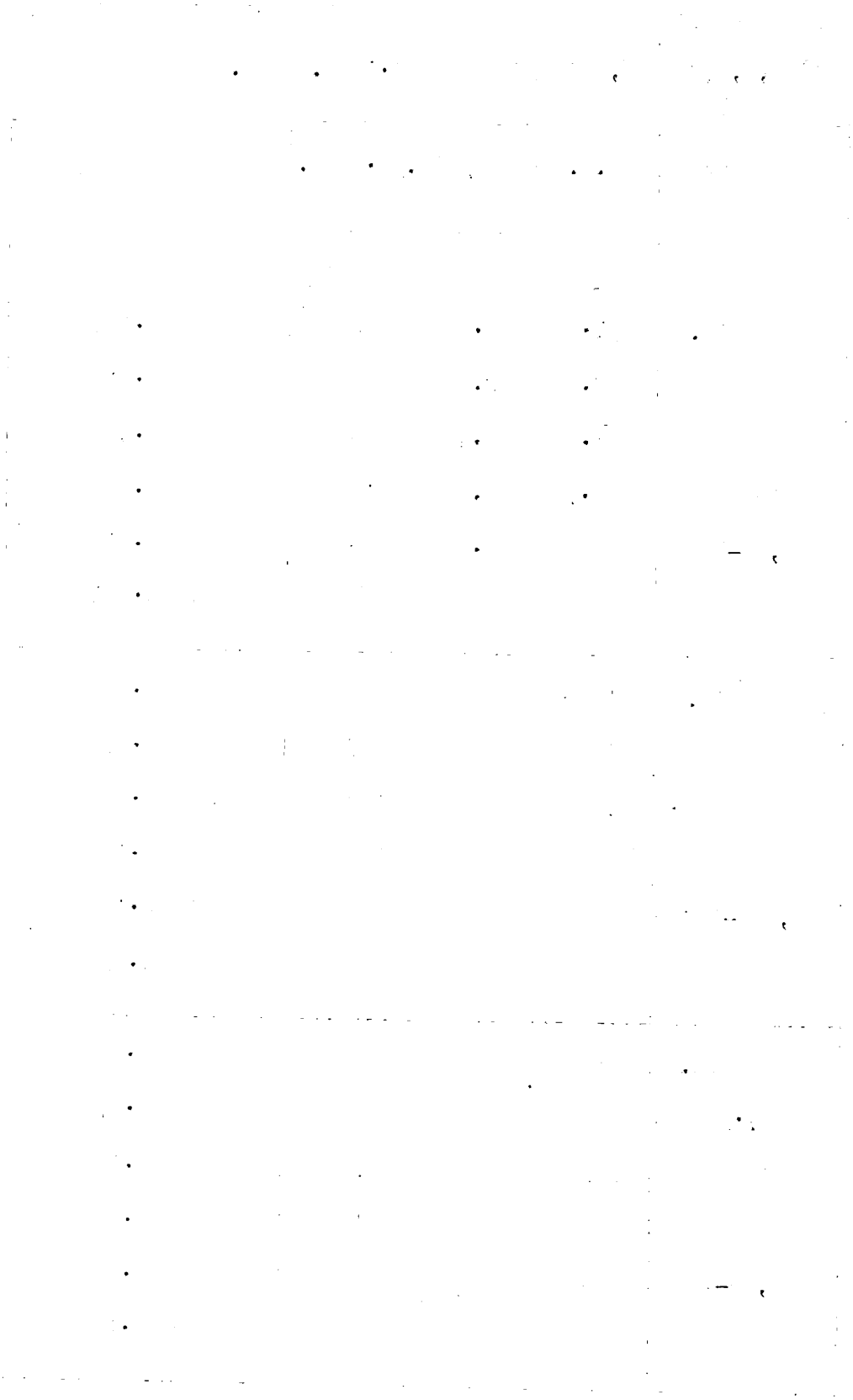


TABLE XVII

Uptake of [^{14}C] DNFB by human red cells during incubation at (1) 21°, (2) 28°, (3) 33° with 1·4 mM. DNFB.

Time	R.A. counts/min./0·2 ml.			Haemoglobin
	Supernatants		Final	Reading
	1	3	Suspension	
(1) 5 min.	51·1	3·8	96	0·475
53	67·4	2·9	320	0·54
120	71·7	9·4	396	0·35
240	75·7	8.0	742	0·58
X, 5-160		3·1	86	0·445
				Standard 0·424
(2) 5 min.	341		143	0·62
20	318		297	0·675
33	272		478	0·82
95	280		530	0·49
X, 10-160	263		166	0·48
				Standard 0·59
(3) 2 min.	329		130	0·44
7·5	330		202	0·27
15	307		376	0·45
60	287		748	0·50
X, 5-160	226		120	0·27
				Standard 0·435

The results of the first three experiments are shown in table XVII. It may be seen that in experiment 1 relatively little radioactivity was lost in the final washing. In subsequent experiments samples were not taken of the final washing. Yields of radioactivity in experiment 1 were lower than expected, and a substantial loss of DNFB by evaporation was suspected. To prevent this, in subsequent experiments a solution of cysteine (30 mg. per 25 ml.) was used as a "fixer": the DNFB-containing sample was mixed with the cysteine solution in the proportion 5:1, or the 0·2 ml. samples were pipetted onto planchets already containing 0·1 ml. cysteine solution. This treatment greatly increased the recovery of radioactivity in the supernatants where the DNFB had previously been free, but made little difference to the recovery in the red cell suspensions where the DNFB was already fixed by the amino-groups of the red cell membrane.

The progressive uptake of DNFB by the red cells may be seen by the results for the final suspensions. Temperature-dependence is shown by the uptake of 742 counts/min. in 240 min. at 21°, and 748 counts/min. in 60 min. at 33°.

Calculations.

Inhibition of glucose transfer was calculated as described by Bowyer & Widdas (1958).

The number of red cells present in each final suspension was calculated from the haemoglobin reading. A standard solution of the blood being used that day was made up to contain 50 µl. blood per 100 ml. Assuming a red cell count of four million cells per µl. blood, the number of red cells in 1 ml. standard = $4 \times \frac{50}{100}$ million per ml.

TABLE XVIII

Uptake of DNFB by human red cells. Results calculated from experimental results shown in table XXII. Red cells were incubated with 1·4 ml. DNFB at (1) 21°, (2) 28° and (3) 33° C.

Time (min.)	% Inhib. of glucose transfer	No. of red cells per 0·2 ml.	Molarity DNFB µmoles./0·2 ml.	Molecules DNFB per red cell
(1) 5	34·5 %	13·5 million	0·0058	2.6×10^8
53	97·5	15·3	0·0192	7.5×10^8
120	98·2	9·9	0·0238	14.3×10^8
240	99·2	16·4	0·0446	16.3×10^8
X, 5-160		12·6	0·0052	2.5×10^8
(2) 5	45·7 %	12·6 million	0·0086	4.4×10^8
10	70·2	13·7	0·0178	6.2×10^8
20	91·5	16·7	0·0286	8.2×10^8
33	96·4	5·0	0·0318	10.6×10^8
95	99·1	9·8	0·0100	20.0×10^8
(3) 2	14·0 %	12·1 million	0·0078	3.9×10^8
7·5	81·6	10·15	0·0121	6.9×10^8
15	89·0	12·25	0·0226	11.0×10^8
60	99·2	13·6	0·0449	19.7×10^8
X, 5-160	70·0	7·5	0·0072	5.8×10^8

The standard sample was directly compared with 0·2 ml. samples diluted 1:6 from the final red cell suspensions. If the reading were the same, then the red cell count in 0·2 ml. suspension would be:-

$$4 \times \frac{50}{100} \times 6 = 12 \text{ million.}$$

Since the red cell counts in the suspensions varied slightly due to slight inaccuracies and losses during the sampling and washing procedures, the haemoglobin readings were used to calculate the correct red cell counts.

The uptake of DNFB was calculated as follows:-

The final incubating mixture contained 5 ml. red cell suspension + 5 ml. DNFB solution. The concentration of DNFB in this solution was 1·4 mM. and the total radioactive counts were 224,000 counts/min. Thus each 1 ml. sample removed from the incubation mixture contained 22,400 counts/min. and 1·4 μMoles.

Therefore 100 counts recovered in the cell suspension is equivalent to:-

$$1{\cdot}4 \times \frac{100}{22{,}400} = 0{\cdot}006 \text{ μmoles. DNFB.}$$

1 μM. DNFB contains 6×10^{17} molecules per litre.

In a suspension containing 10 million red cells, the number of DNFB molecules present would be:-

$$\frac{0{\cdot}006 \times 6 \times 10^{17}}{10 \text{ million}} = 3{\cdot}6 \times 10^{8} \text{ per red cell.}$$

The results of the calculations are shown in table XVIII.

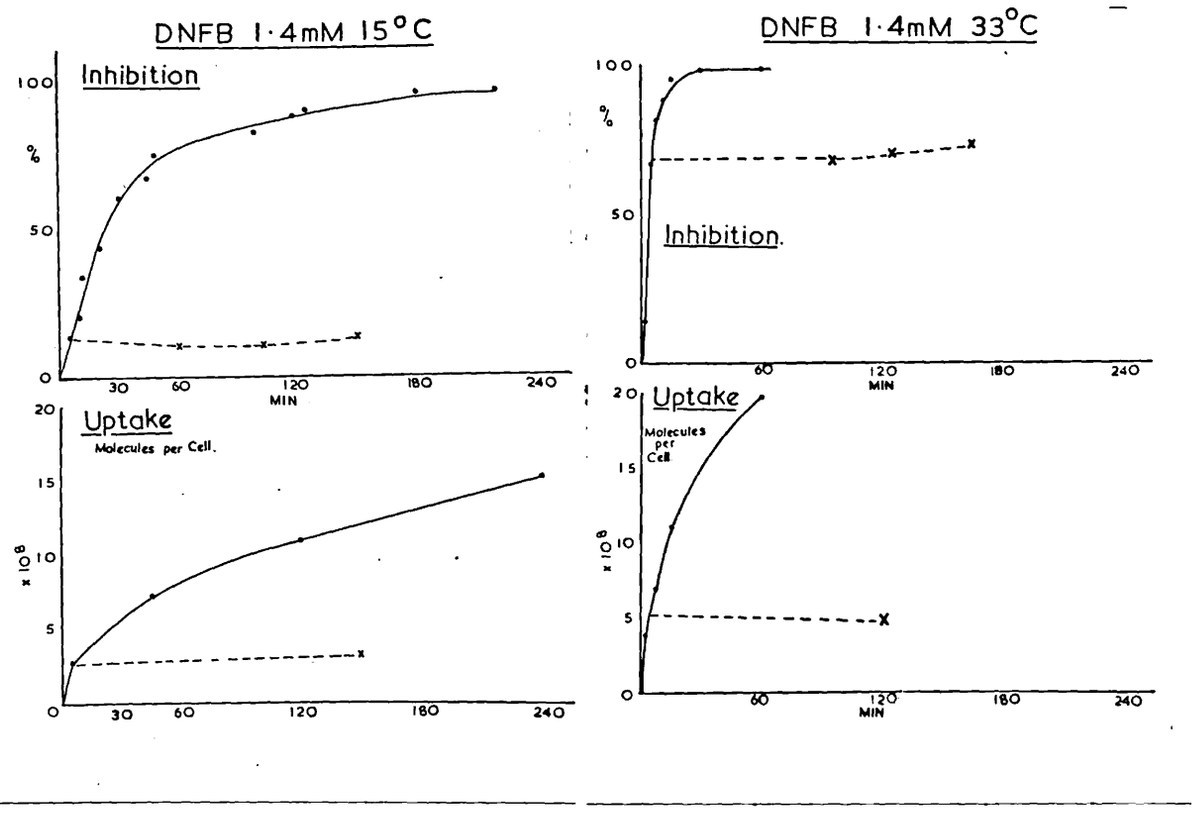


Fig. 19. Inhibition of glucose transfer in human red cells by 1·4 mM DNFB. Above: time-course of inhibition; below: time-course of uptake of DNFB in molecules per red cell:

(1) at 15°; (2) at 33°.

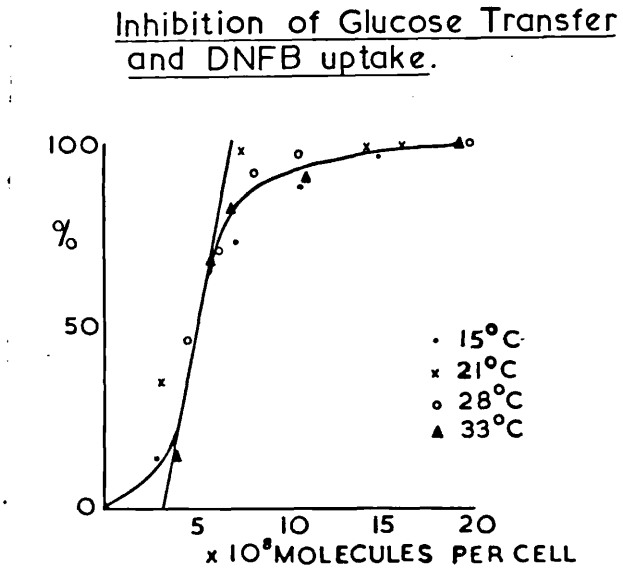


Fig. 20. Inhibition of glucose transfer in human red cells by 1.4 mM DNFB. Percentage inhibition is plotted against uptake of DNFB in molecules per red cell.

In a similar experiment, carried out at 15°, the following results were obtained:-

Time	% Inhibition	Uptake of DNFB
5 min.	13.5%	2.76×10^8 molecules per red cell
45 min.	72.5%	7.2×10^8 molecules per red cell
120 min.	88.0%	10.8×10^8 molecules per red cell
240 min.	95.9%	15.0×10^8 molecules per red cell
X, 5-160 min.	13.5%	3.0×10^8 molecules per red cell

Graphs were drawn to show the time-courses of inhibition of glucose transfer and of uptake of DNFB at 15° and 33°, and they are shown in fig. 19. The dotted lines show the results obtained for samples X, i.e. those red cells removed from the incubation mixture at an early stage and re-incubated in a DNFB-free medium. These results demonstrate that both inhibition and uptake are irreversible.

The solid lines show the progressive inhibition and uptake when the red cells were incubated in the presence of DNFB. Both processes were slower at the lower temperature, but the relation between inhibition and uptake is independent of temperature, as may be seen from fig. 20, which summarises all the results obtained in the present experiments.

The steepest part of the graph in fig. 20 gives a slope which suggests that an uptake of about 400 million molecules of DNFB per red cell would be required to produce full inhibition of glucose transfer. However, this must be regarded as an over-estimate of the number of membrane carrier-sites, since it cannot be assumed that DNFB is attacking only those groups concerned in sugar transfer.

It is of some interest to note that LeFevre et al. (1964) found that a hot ethanol-ether extract of red cells could be loaded with glucose or other monosaccharides up to about 500 million molecules per red cell, under completely unphysiological conditions, and that the weak association between hexose and extract thus formed was easily broken by the addition of water to the dry extract. However, LeFevre (1961) calculated that the upper limit of the number of transfer sites in the human red cell ghost must be 500,000. This figure seems rather low, but his calculation was based on a method which is open to some criticism, particularly since it has been impossible to show in this laboratory that red cell ghosts are still capable of facilitated glucose transfer.

Uptake of [^{14}C] DNFB by Human Red Cell Lipids.

In the fourth experiment of the present series, the red cells were extracted with n-butanol after incubation with [^{14}C] DNFB and the lipid extract was separated to localise the [^{14}C]-labelled components.

Method. Time-expired red cells in which the facilitated transfer mechanism was still intact were treated as follows:-

50·0 ml. packed red cells
92·5 ml. buffered saline
7·5 ml. 30% glucose

were mixed and equilibrated at 37° for 30 minutes, cooled to 33°, then the following mixture added:-

16·5 ml. alcoholic solution [^{14}C] DNFB, giving a final concentration of 1·4 mM.
7·5 ml. 30% glucose
126·0 ml. buffered saline.

Samples were taken from time to time to measure the inhibition of glucose transfer. At 60 minutes, inhibition was almost complete. The mixture was spun, and 48 ml. red cells recovered. These were added to 480 ml. n-butanol, slowly, swirling the contents of the flask throughout, and left to extract overnight at 4^{o}.

No aqueous layer separated out, and the total extract was filtered.

100 ml. methanol was added to the remaining solids, shaken well and filtered.

To the solids still remaining, 190 ml. ethanol and 20 ml. conc. HCl + 30 ml. water was added, shaken and allowed to stand at room temperature for 2 hours. Then 250 ml. 0·9% NaCl was added, shaken, and the mixture was stood at 4^{o}.

0·2 ml. samples of each of these extracts were taken, and the total counts in each extract calculated. The following results were obtained:-

Extract	Volume recovered	R.A. counts/0·2 ml.	Total counts
Butanol	460 ml.	66·2/min.	152,300/min.
Methanol	100 ml.	38·0/min.	19,000/min.
Acid-alcohol	500 ml.	150·0/min.	375,000/min.

The total radioactivity added to the mixture was calculated from a standard to be 6,748,000 counts/min. The percentage recovery of radioactivity in each extract was thus:-

Butanol	2·26%
Methanol	0·28%
Acid-alcohol	5·58%
Remainder	91·9% still remaining in the solid residue, which was largely haemoglobin.

The concentration of the [^{14}C] DNFB standard was 0·28 mM., and its radioactivity 850 counts/0·2 ml.

The molarity of the butanol extract was thus:

$$0{\cdot}28 \times \frac{66{\cdot}2}{850} \times 1000 = 21{\cdot}8\ \mu M.$$

Total number of μmoles in 460 ml. extract = $21{\cdot}8 \times \frac{460}{1000}$

= 10·1 μmoles per 50 ml. red cells.

Expressed in molecules of DNFB, this is 10 to 20 million per red cell.

It is interesting to compare this result with those of other workers. LeFevre (1961), using a method of incubating ghost suspensions in low concentrations of (^{14}C] glucose, then spinning, calculated the number of sites concerned in glucose transfer could not be greater than 500,000 per ghost, and he assumed that the result for intact red cells would be similar. However, in this laboratory it has not been possible to show satisfactorily that the transfer mechanism still operates in red cell ghosts. Weed, van Steveninck and Rothstein (1964) used a number of mercurial compounds to inhibit glucose transfer in red cells, presumably by attacking the sulphydryl groups. They found the -SH groups to be distributed in the red cell as follows: haemoglobin 90%, glutathione 6%, membrane 4%. Glucose transfer was inhibited when 1·2% of the membrane -SH groups had reacted with parachloromercuribenzoate (PCMB), and from this they calculated that the maximum number of transport sites in the intact red cell is 700,000 per cell, and that this number of glucose molecules would cover about 1% of the red cell surface. Whether PCMB will attack other groups, as will DNFB, was not made clear.

From these results it seemed likely that the amount of DNFB recovered in the lipid extracts was enough, possibly 20 times more than enough, to account for the glucose transfer sites if these had indeed been extracted during the method described.

The experiment was continued as described below.

The butanol extract was evaporated down at reduced pressure under a stream of nitrogen at 50-60° to dryness. The dried extract was taken up in chloroform, filtered, and the little insoluble residue taken up in carbon tetrachloride. This probably consisted of protein material extracted by butanol but insoluble in chloroform; it contained some radioactivity.

138 mg. chloroform-soluble extract was obtained, with total radioactivity 51,200 counts/min.

Since the total radioactivity in the butanol extract was originally 152,300 counts/min., two-thirds of the [^{14}C] DNFB had been lost, probably largely by evaporation at the rather high drying temperature. This suggests that much of the DNFB attached to the lipids was not in chemical combination but forming a reversible complex.

50 mg. of the chloroform-soluble extract was fractionated on a silicic acid column in the usual way by elution with chloroform-methanol 9:1, 4:1, 3:2 and 1:4, and samples were taken of each fraction for radioactive counts. The fractions were then pooled to give measurable dry weights (by evaporation at below 37°), and each pooled group dried and taken up in, first, carbon tetrachloride, second, carbon disulphide, for infra-red spectroscopy. It was found that DNFB could not be detected by infra-red spectroscopy, but that the distribution of lipids was similar to previously described fractionations.

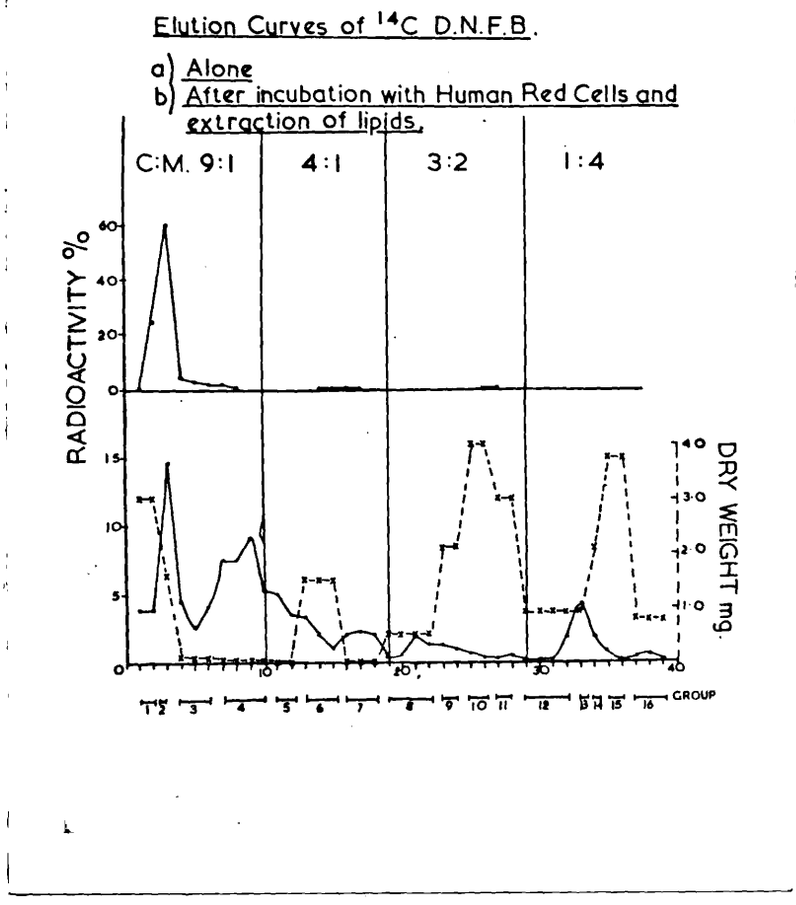


Fig. 21. Uptake of [^{14}C] DNFB by human red cell lipids. Red cells extracted with n-butanol and 50 mg. extract eluted on a silicic acid column.

(a) Elution of [^{14}C] DNFB alone.

(b) Elution curves of radioactivity and dry weights in lipid extract.

The elution curve showing recovery of radioactivity and dry weights is shown in fig. 21.

[^{14}C] DNFB, when applied to a column alone, is eluted almost completely in the early part of chloroform-methanol 9:1, as is also shown in fig. 21.

On thin-layer chromatograms, DNFB, when applied alone, travels with the solvent front.

The pooled lipid fractions, after being used for infra-red spectroscopy, were applied to thin-layer plates and developed and stained in the usual way. Those spots with a total of at least 700 counts/min. were scanned for radioactivity.

Groups 1 and 2 (fractions 1 - 3) showed a radioactive peak at the solvent front, which was either free DNFB or DNFB associated with fast-travelling lipids such as glycerides and sterols. Groups 3 and 4 (fractions 4 - 10) showed peaks at the solvent front and between Rf 0·15 and 0·3. Groups 6 and 7 (fractions 13 - 18) showed ninhydrin positive radioactive peaks between Rf 0·2 and 0·3, and group 13 (fraction 33) a ninhydrin negative peak at about Rf 0·1. The peaks in groups 6 and 7 were probably associated with phosphatidylserine and phosphatidyl-ethanolamine, that in group 13 with sphingomyelin or lysophosphatides.

Some radioactive peaks appeared to be associated with non-phosphorus-containing components. These could have been either lipids such as cerebrosides which are known to associate with phospholipids, or possibly such substances as lipo-amino acids or phosphopeptides which might have been extracted in the procedures used. No attempt was made at this stage to identify the radioactivity-containing components.

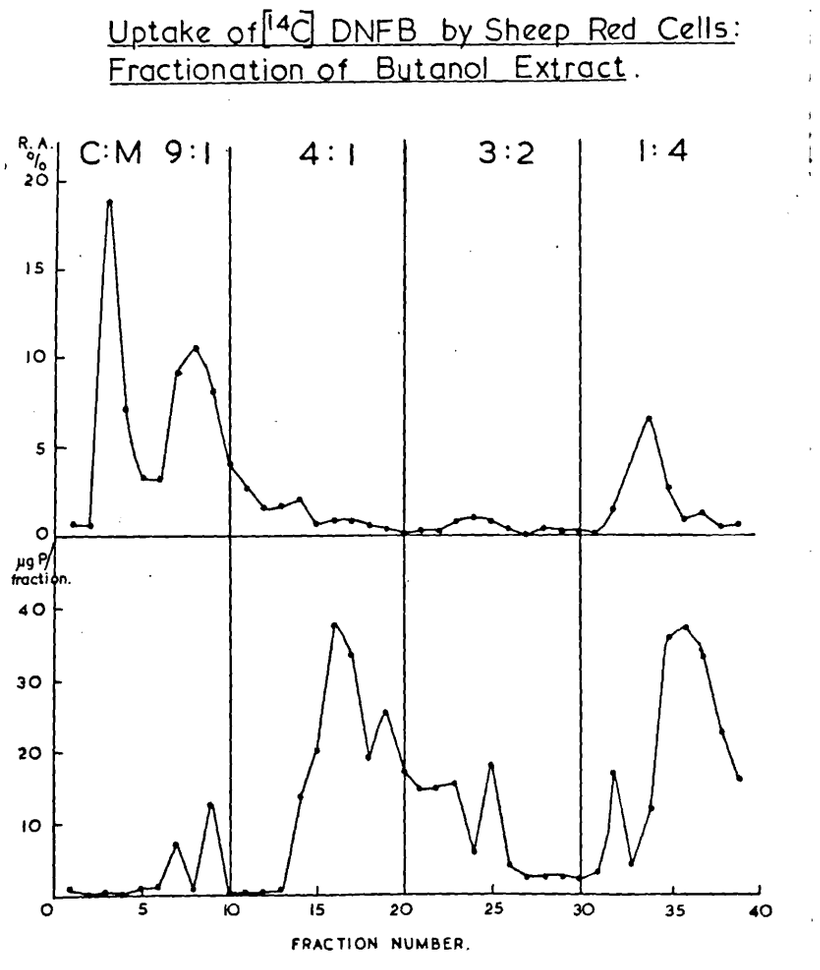


Fig. 22. Elution curves of sheep red cell lipids, incubated with [^{14}C] DNFB and extracted with n-butanol. (Experiment A)

(1) Counts per fraction, % of total.

(2) Phosphorus content of fraction, µg. P per fraction.

The next stage was to repeat the incubation and extraction with fresh human blood, in case the carrier substance is susceptible to degradation processes, and to compare these results with those obtained from a parallel experiment using sheep red cells.

A Comparison of [^{14}C] DNFB Uptake by Human and Sheep Red Cell Lipids.

A. Sheep. Sheep blood was obtained from St. Mary's Hospital Medical School two days beforehand, and spun and washed to give 30 ml. red cells. They were treated as follows:-

30 ml. red cells,

4·1 ml. 30% glucose solution,

47·7 ml. buffered saline

were incubated at 33° for a few minutes, then the following mixture, also at 33°, was added to it:-

9 ml. alcoholic solution DNFB, containing 45 mg., part of it [^{14}C] labelled,

4·1 ml. 30% glucose solution,

68·7 ml. buffered saline.

The two mixtures were incubated together for 1 hour at 33°, then the red cells were spun down and added slowly to 300 ml. n-butanol, mixed thoroughly, and left to extract overnight at 4°.

The extract was filtered and the remaining solids were discarded. The radioactivity was distributed as follows:-

Total R.A. added to incubation mixture	1,310,850 counts/min.	
R.A. recovered in lipid extract	36,000 counts/min.	
% Recovery	2·7%	
R.A. recovered in incubation supernatant	53,560 counts/min.	= 4%
∴ R.A. remaining in red cell solids	1,150,000 counts/min.	= 94%

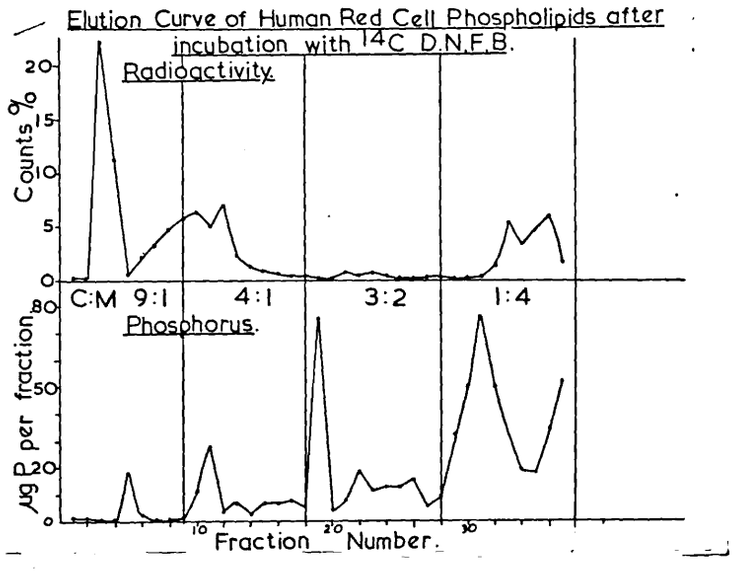


Fig. 23. Elution curves of human red cell lipids, incubated with [^{14}C] DNFB and extracted with n-butanol. (Experiment B)

(1) Counts per fraction, % of total.

(2) Phosphorus content of fraction, µg. P per fraction.

207 mg. dry lipid extract was obtained. It was taken up in chloroform, and 50 mg. was fractionated on a silicic acid column. The fractions were analysed for phosphorus content, radioactivity, and pooled to obtain the dry weights. The results are shown in fig. 22.

B. Human Red Cells. Venous blood was obtained from J.G. and J.R., and washed and spun immediately. 30 ml. red cells were recovered and were treated as described for the sheep red cells. The following results were obtained:-

Total R.A. added	1,310,850 counts/min.		
R.A. in lipids	47,000 counts/min.	=	3·6%
R.A. in supernatant	44,000 counts/min.	=	3·3%
∴ R.A. in solids	1,220,000 counts/min.	=	87%

193 mg. dry lipid extract was obtained. 50 mg. was fractionated and the elution curves are shown in fig. 23.

The elution curves of radioactivity obtained for sheep and human red cells were similar, the main difference being that a substantial proportion was obtained in fractions 7-9 in sheep, associated with a small phosphorus peak, while in the human the radioactivity extended between fractions 9-13, forming a double peak, and this was associated with the early phosphorus peak in the chloroform-methanol 4:1 as well as the later fractions of 9:1. Some of the differences in the phosphorus peaks were as expected, as it is known that sheep red cells contain considerably less lecithin and more sphingomyelin than human red cells.

The results obtained of radioactivity, phosphorus and dry weights for each solvent mixture were pooled so that an overall comparison could be made. The results are shown in table XIX..

TABLE XIX

Sheep and Human Red Cell Lipids after Incubation with [^{14}C] DNFB: a Comparison of Recoveries of Radioactivity, Phosphorus and Dry Weights eluted by Chloroform-Methanol Solvent Mixture.

A. SHEEP

Solvent Mixture	µg.P	R.A. Cnts./min.	Dry Weight
(1) Chloroform-Methanol 9:1	28•5	4,726	14•5 mg.
(2) Chloroform-Methanol 4:1	166•3	833	7•4 mg.
(3) Chloroform-Methanol 3:2	74•4	347	20•5 mg.
(4) Chloroform-Methanol 1:4	185•3	1,395	14•6 mg.
Total	454•5	7,301	57•0 mg.

B. HUMAN

Solvent Mixture	µg.P	R.A. Cnts./min.	Dry Weight
(1) Chloroform-Methanol 9:1	37•4	4,206	8•4 mg.
(2) Chloroform-Methanol 4:1	145•0	1,336	11•4 mg.
(3) Chloroform-Methanol 3:2	91•9	230	19•0 mg.
(4) Chloroform-Methanol 1:4	327•5	1,645	4•2 mg.
Total	601.8	7,417	43•0 mg.

It may be seen that although a slightly larger dry weight of sheep red cell lipids was applied to the column, a rather lower yield of phosphorus was obtained. A higher proportion of cholesterol in the sheep was suspected, and infra-red spectroscopy showed this to be the case. The total radioactivity in the sheep was slightly lower than in the human red cells, but this was probably not so important as the different distributions between solvents 1 and 2.

The lipids of the various peaks were separated by thin-layer chromatography, using sphingomyelin, phosphatidylinositol, cerebrosides and a commercial preparation of cardiolipin, containing largely cholesterol and lecithin, as reference markers. The peaks with a high radioactivity content were scanned.

In the sheep red cell lipids, the radioactivity in the first peak was found to be partly fast-moving, and thus either free or accompanying the fast-moving sterols and glycerides, and partly in a slower component which was also positive to iodine. In the second peak (fractions 7-9), radioactivity was found associated with a comparatively fast-moving component, positive to iodine and rhodamine, containing phosphorus, but negative to ninhydrin. The identity of this component was unknown. (Ninhydrin staining is of little relevance in these chromatograms since, if the free amino-groups have already reacted with DNFB, they will not give a positive reaction with ninhydrin.)

In the human red cell lipids, radioactivity in the first peak was fast-moving. In the second peak (fractions 5-9), radioactivity was associated with iodine-positive relatively fast-moving components, about

level with the cerebroside marker. In the third peak (fractions 10-12) there were two radioactive components which appeared to correspond to phosphatidylethanolamine and phosphatidylserine. In the last peak some radioactivity was found associated with sphingomyelin.

If the hypothetical membrane carrier is indeed a phospholipid, and is thus presumably present in human red cells to a greater extent than in sheep red cells, and if the DNFB exerts its inhibitory action by reacting irreversibly with the carrier component (while simultaneously reacting with other components in a non-discriminating fashion), then the part of the elution curve of the human red cell phospholipids of greatest interest appears to be the peak eluted early in chloroform-methanol 4:1, that is, the peak usually associated with the cephalins, phosphatidyl-ethanolamine and phosphatidylserine.

The rest of the human red cell extract, which had been stored at 4^{o} in an atmosphere of nitrogen while the work described above was being performed, was fractionated on silicic acid columns, giving elution curves very similar to those already described. The third peak (early 4:1) from each of the columns was pooled, to obtain enough material to study further the nature of the components and with the hope of identifying the DNP-amino-acids present.

This further analysis was performed by Miss Mary L. Forsling and the procedure was briefly as follows. The lipids were chromatographed in broad bands, and the positions of the DNP-amino-lipids, while often immediately visible due to the characteristic yellow colour of DNP-compounds, were confirmed by scanning the radioactivity. The area

containing the DNP-lipids was marked out and the silica within that area scraped free from the glass with a scalpel. The loose silica was sucked into a glass aspirator, then chloroform-methanol 4:1 injected into the aspirator to elute the lipids from the silica. The apparatus and method are described by Goldrick and Hirsch (1964). The lipids were then hydrolysed for 16 hours at 110° with 5N HCl, and the hydrolysate was divided into an ether-soluble portion, a butanol-soluble portion and a water-soluble residue containing only free amino-acids. The first two portions were run on two-way chromatograms using known DNP-amino-acids as markers (Biserte, Holleman, Holleman-Dehove & Soutiere, 1960).

The ether-soluble portion gave a yellow radioactive spot which could have been glycine, lysine, serine or ethanolamine, and the butanol-soluble portion gave a positive reaction but the component could not be identified.

Experiment C in this series was performed using human time-expired blood and a higher proportion of [^{14}C] DNFB. The following results were obtained:-

(Results for fresh human blood, experiment B, are shown in parentheses.)

	C.	B.
R.A. added to red cells	7,474,500 counts/min.	(1,310,850)
R.A. recovered in butanol extract	127,125 counts/min.	(47,000)
Percentage	1·8%	(3·6)
R.A. in chloroform-soluble lipids	32,745 counts/min.	(29,668)
Percentage	0·45%	(2·3)

Thus the addition of six times the amount of [^{14}C] DNFB raised the uptake in chloroform-soluble lipids by only 10%. Assuming that the uptake

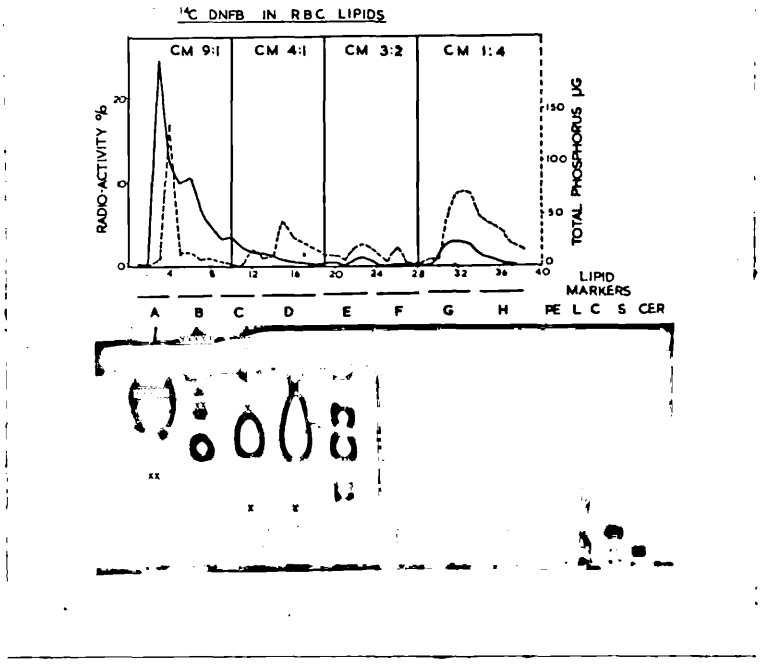


Fig. 24. Uptake of [^{14}C] DNFB by human red cell lipids. (Time-expired, Experiment C)

Above: elution curve of butanol-extracted, chloroform-soluble lipids.

Below: thin-layer chromatograms of the pooled peaks.

The reference markers were as follows:-

K: synthetic phosphatidylethanolamine. L: synthetic phosphatidyl-choline. Card: commercial cardiolipin. S: sphingomyelin.

Cer: cerebrosides.

of DNFB by the lipids follows a similar curve to the inhibition-uptake curve shown for whole red cells in fig. 20, this suggests that the quantity of [^{14}C] DNFB used for the earlier experiment was ample to saturate any lipids involved in glucose uptake.

Not all the solids obtained in the butanol extract were chloroform-soluble. The chloroform-insoluble portion, a pale yellow powder, was fairly high in radioactivity and gave positive protein reactions, suggesting that it was proteolipid or protein in nature. It was not thoroughly investigated although preliminary tests showed it to be of possible interest.

The chloroform-soluble lipids from time-expired human blood were separated on a silicic acid column in the usual way, and their elution curve is shown in fig. 24. The fractions in each peak were pooled as indicated, and separated further on the thin-layer chromatograms as shown below. The crosses on the spots indicate the strength of radioactivity present in the more active spots.

The spot corresponding to phosphatidylethanolamine gave, on hydrolysis and separation, a DNP-amino-acid which was possibly DNP-ethanolamine. A free amino-acid was isolated from the other spots which was not identified.

These three experiments, in which sheep and human red cells were incubated with [^{14}C] DNFB, succeeded in that some lipids were labelled irreversibly in quantities large enough for further analysis. The positions of these lipids in the elution curves were not exactly as expected, being eluted earlier than those labelled with [^{14}C] glucose. They appeared to be similar in nature, that is, of the cephalin type, so it may be that the presence of DNFB increases the mobility of the cephalins.

It has already been noted that DNFB inhibits glucose transfer in the red cell more effectively when glucose is present, and for this reason glucose was added to all the [^{14}C] DNFB incubating mixtures in the previous set of experiments. It was not known whether DNFB acted by superimposing itself upon the glucose molecule at the transport site or by displacing it. If it superimposed itself, there is the possibility that the glucose would then be fixed as irreversibly as the DNFB.

To attempt to answer these two questions, that is, of phospholipid mobility and of glucose fixation, another pair of experiments was performed.

The Effect of DNFB on Uptake of [^{14}C] Glucose.

The purpose of these experiments was to compare the uptake of glucose in the absence and presence of DNFB.

In the first experiment, with [^{14}C] glucose alone, incubation time was limited to 60 seconds at a low temperature to minimise metabolic incorporation of glucose. In the second, the red cells were first equilibrated with non-radioactive glucose at 37^{o} to saturate the glucose carrier mechanism and the glucose metabolic pathways, then incubated with [^{14}C] glucose for 20 seconds before adding DNFB and incubating for a further 60 seconds. It was hoped that the high concentration of DNFB used would bring about a substantial inhibition in this short time and that metabolic incorporation of [^{14}C] glucose would again be minimised.

Human Red Cells, time-expired, were washed three times with buffered saline and tested for glucose exits.

^{14}C Glucose, 15 μc./ml., 176,000 counts/min./μc.

DNFB, non-radioactive, in 50% alcohol, to give final concentration of 10 mM.

TABLE XX

Uptake of [^{14}C] Glucose by Human Red Cells in the Absence and Presence of DNFB.

	Dry Weights	R.A. Counts
(1) Without DNFB		
[^{14}C] glucose added		3,520,000
Chloroform-soluble lipids	215 mg.	328,700
Chlor-meth. 1:1 soluble lipids	7	36,100
"Insoluble powder"	8	8,660
Total	230	373,460
Recovery		10.6%
(2) With DNFB		
[^{14}C] glucose added		4,400,000
Chloroform-soluble lipids	140 mg.	34,550
Chlor-meth. 1:1 soluble lipids	135	156,280
"Insoluble powder"	90	193,780
Total	365	384,610
Recovery		8.75%

(1) Without DNFB. 4 ml. [^{14}C] glucose solution was added to 50 ml. ice-cold red cells, shaken for 20 seconds, then poured into 500 ml. cold n-butanol, stirred, and left to stand overnight at 4°.

(2) With DNFB. 50 ml. red cells were equilibrated with 76 mM. non-radioactive glucose at 37° for 30 min. 5 ml. [^{14}C] glucose solution was added, shaken for 20 seconds, then 10 mM. DNFB added and shaken for 60 seconds, then poured into 500 ml. cold n-butanol, stirred and left to stand overnight at 4°.

At 80 seconds, a small portion of the incubation mixture was removed to measure the time course of glucose exits. Inhibition was found to be 15-20%.

The extracts were filtered and evaporated to near dryness under nitrogen. The chloroform-soluble lipids were obtained by adding chloroform to the slightly wet extract, mixing gently and spinning. The residue after decanting was mixed with chloroform-methanol 1:1 and spun. There still remained an insoluble residue which was suspended in carbon tetrachloride and which dried to a white powder. The dry weights and radioactive counts are shown in table XX .

These results show several interesting features. From the dry weights it may be seen that the DNFB-containing extract was less chloroform-soluble but contained a greater amount of chloroform-methanol soluble and residual material. It appears from this that the addition of DNFB renders the extraction of proteolipids by hutanol more efficient and that as a result there is a greater amount of protein-like insoluble powder remaining behind after the lipid moiety has been solubilised. The high

radioactivity of the insoluble powder shows that it includes a considerable proportion of the [^{14}C] glucose, either as the free sugar after being displaced by DNFB during the extraction procedure, or in combination with a carrier component which has been rendered chloroform-insoluble by the presence of DNFB. It probably includes much of the non-radioactive sugar added at the beginning of the incubation as well. The total uptake of [^{14}C] glucose was similar in the two experiments and the percentage recoveries were very high: in the first experiment this was probably the result of keeping the temperature low, where kinetics would favour the association but not the dissociation of the glucose-carrier complex; in the second it was presumably due to the presence of DNFB.

The extracts were separated on silicic acid columns as follows:-

(1) Chloroform-soluble lipids from the extraction without DNFB. 210 mg. was separated on 20 g. silicic acid + 10 g. Hyflo-supercel, and eluted with these mixtures:

100 ml.	chloroform-methanol	9:1	and	collected	in	10 ml.	fractions
100 ml.	"	11:2	"	"	"	10 ml.	"
100 ml.	"	4:1	"	"	"	10 ml.	"
150 ml.	"	3:2	"	"	"	15 ml.	"
150 ml.	"	1:4	"	"	"	15 ml.	"

(2) Chloroform-soluble lipids from extraction with DNFB. 130 mg. was separated on 15 g. silicic acid and 7·5 g. Hyflo-supercel and eluted as in (1).

(3) Chloroform-methanol 1:1 soluble lipids from extraction with DNFB. 75 mg. (the other 60 mg. was no longer soluble) was separated on 13·5 g.

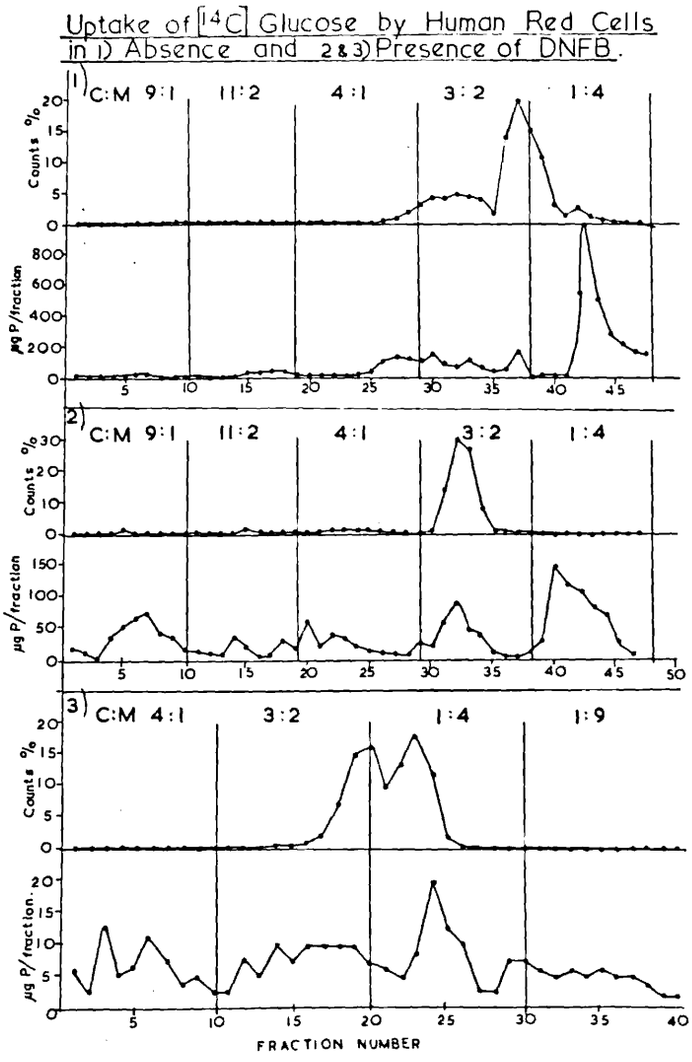


Fig. 25. Elution curves of human red cell phospholipids, incubated with [^{14}C] glucose, then extracted with n-butanol:

(1) without DNFB, chloroform-soluble lipids;

(2) with DNFB, chloroform-soluble lipids;

(3) with DNFB, chloroform-methanol 1:1 lipids.

TABLE XXI

Uptake of [^{14}C] Glucose by Human Red Cells in the Absence & Presence of DNFB.

Separation of butanol-extracted lipids on silicic acid-Hyflo-supercel columns.

Solvent Mixture		R.A. cnts./min.	µg.P	Dry Wt.
(1) Chloroform-soluble lipids, without DNFB				
Chloroform-methanol	9:1	1,670	92	40·6 mg.
" "	11:2	1,915	144	4·8
" "	4:1	32,930	693	16·0
" "	3:2	248,300	649	34·0
" "	1:4	30,270	1,928	62·3
Total		315,085	3,506	157·7
(2) Chloroform-soluble lipids, with DNFB				
Chloroform-methanol	9:1	1,370	308	56·7 mg.
" "	11:2	1,760	139	10·0
" "	4:1	3,160	245	5·8
" "	3:2	40,940	318	20·8
" "	1:4	861	648	22·1
Total		49,091	1,658	115·4
(3) Chloroform-Meth. 1:1 sol. lipids, with DNFB				
Chloroform-methanol	4:1	655	62·5	2·0 mg.
" "	3:2	48,000	80·0	22·0
" "	1:4	35,500	82·5	31·0
" "	1:9	815	47·5	1·6
Total		84,970	272·5	56·6

silicic acid and 6·8 g., and eluted with 100 ml. each of the mixtures chloroform–methanol 4:1, 3:2, 1:4 and 1:9, and collected in 10 ml. fractions.

Table XXI shows the results for each solvent mixture. Fig. 25 shows the elution curves. Chloroform–methanol 11:2 was used as the second solvent mixture in (1) and (2) to separate the cardiolipin from the cephalins: some lipid was obtained in these fractions, but the relative radioactivity was no higher than that of the cephalins. Chloroform–methanol 1:9 was added as a final solvent mixture to (3) in order to elute the more highly polarised lipids which might have been present. These fractions also had a low radioactivity.

Thin-layer chromatography of the various peaks gave results consistent with previous experiments.

(1) Chloroform-soluble lipids of [^{14}C] glucose incubation of red cells without DNFB. The low, wide peaks in late chloroform–methanol 4:1 and early 3:2 separated into several spots, of which at least two were ninhydrin-positive with Rf's corresponding to the cephalin markers. Radioactivity was found in the Rf region 0·5 – 0·7, associated with the amino-group containing lipids. In the large peak of late chloroform–methanol 3:2, the radioactivity stayed near the origin and was thus probably free glucose. The radioactivity peak in 1:4, appearing as a shoulder of the 3:2 peak, separated to a spot in the Rf region 0·2 – 0·4, associated with phospholipid which was probably lysophosphatide.

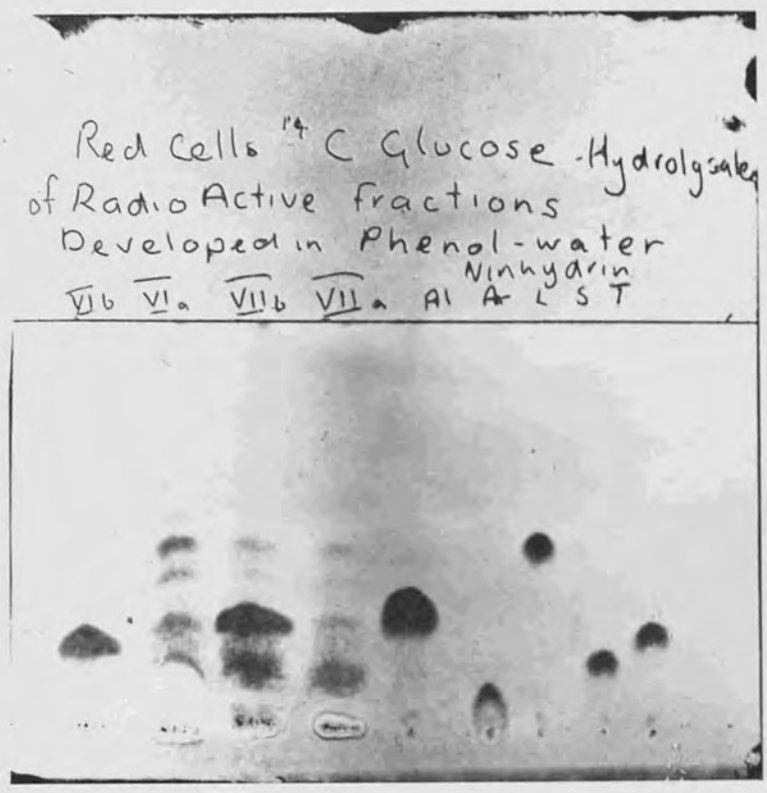


Fig. 26. Thin-layer chromatogram of phospholipid hydrolysates, stained for amino-acids.

"Cephalin" peak from butanol extract of red cells previously incubated with [^{14}C] glucose in the absence of DNFB, hydrolysed with HCl, developed in phenol-water, stained with ninhydrin.

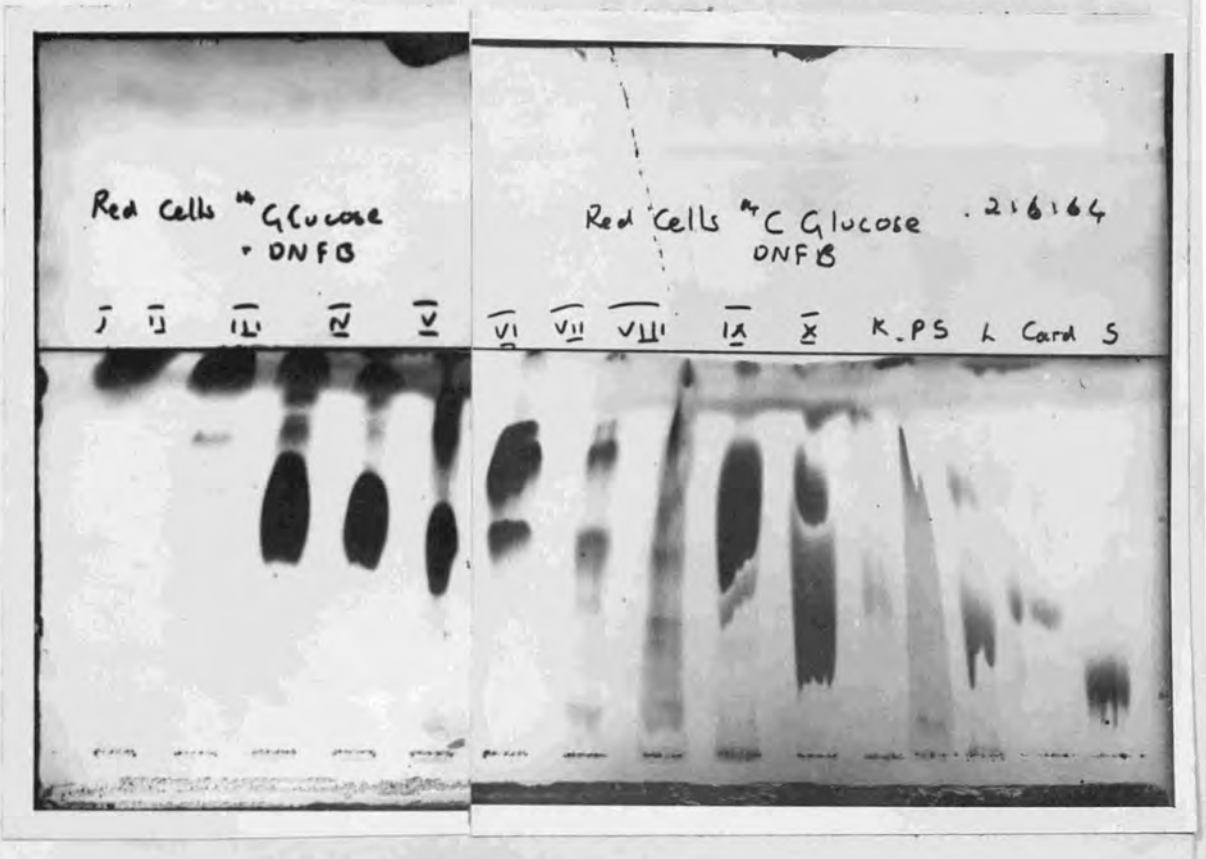


Fig. 27. Thin-layer chromatograms of chloroform-soluble lipids from red cells incubated with [^{14}C] glucose and DNFB. The pooled fractions were eluted with chloroform-methanol as follows:-

I & II - early and late 9:1; III & IV - early and late 11:2;

V & VI - early and late 4:1; VII & VIII - early and late 3:2;

IX & X - early and late 1:4.

Reference markers: K - cephalin; PS - phosphatidylserine; L - lecithin; Car - cardiolipin; Cer - cerebrosides; S - sphingomyelin.

The fractions comprising the cephalin peak were removed from the plates and hydrolysed, and analysed for amino-acid content by further thin-layer chromatography. The result is shown in fig. 26. At least five amino-acids were present, but not enough radioactivity remained to determine which had been associated with the [^{14}C] glucose. They were separated further by two-way thin-layer chromatography, but definite identifications were not made.

(2) Chloroform-soluble lipids of [^{14}C] glucose incubation of red cells with DNFB. The thin-layer chromatogram of fractions from this separation is shown in fig. 27. Several differences were noted between the results for (1) and (2). First, none of the radio-activity in (2) remained near the origin, although in the peaks eluted by chloroform-methanol 9:1 and 11:2 radioactivity was found at Rf 0·1, associated with spots giving a weak lipid reaction but pale yellow due to the presence of DNP derivatives. Second, cephalins were found in the elution fractions from chloroform-methanol 11:2, 4:1 and early 3:2, and in the early part of the 3:2 peak radioactivity was found at Rf's 0·2, 0·3, 0·5 and 0·85. In the later part of the 3:2 peak, radioactivity was associated with spots at Rf 0.15 and 0.25 which gave lipid reactions and which were possibly inositides. The peak eluted by chloroform-methanol 1:4 contained lecithin and sphingo-myelin, but very little radioactivity. The spots containing high radioactivity were eluted, hydrolysed and tested for amino-acids, and the spots obtained were assigned tentatively to alanine, ethanolamine, glycine and lysine: their identities were not verified.

(3) Chloroform–methanol 1:1 soluble lipids from [^{14}C] glucose incubation of red cells with DNFB. The peaks containing radioactivity were those eluted with chloroform–methanol 3:2 and 1:4. In the late part of 3:2 lecithin and some cephalin was present; the radioactivity was partly near the origin and probably free, and partly associated with the earlier phospholipid spots, at Rf 0·2. The lipids eluted in the 1:4 peak were not identified but were probably lysophosphatides. The radioactive spots were removed, hydrolysed and analysed for amino-acids: none were found.

These results throw a little light on the questions originally posed concerning the stability of the glucose–carrier complex in the presence of DNFB, and the effect of DNFB on the mobility of the cephalins. The results show that, both in the absence and presence of DNFB, [^{14}C] glucose becomes associated with the phospholipids of human red cells, particularly the cephalins, in a form stable enough to separate detectable amounts.

The presence of DNFB increased the uptake of [^{14}C] glucose by the red cells to a level comparable with that in the ice-cold incubation mixture. There was no evidence of DNFB in the highly radioactive fractions of (2) and (3), but if it were present in a quantity comparable with the complexed [^{14}C] glucose then it would probably not be detectable.

The effect of DNFB on cephalin mobility during column chromatography does not appear to be a simple accelerating one. The cephalins in (2) and (3), particularly (2), appeared to be scattered over a much wider elution range than in (1). It is not known whether this was brought

about by the extraction of a wider variety of cephalins in the presence of DNFB, or whether the varying mobilities are related to the extent with which they have reacted with DNFB. More specific spray tests for phospholipids such as the phosphoinositides and cardiolipin would be helpful in identification.

From the variety of amino-acids obtained on hydrolysis of the cephalin fractions it appears that the term "cephalin" may cover a wider range of amino-group-containing phospholipids than was previously realised. Alternatively, there may be present in the extract lipo-amino acids of the type described by Macfarlane (1962), that is, complexes between amino-acids and lipids which retain the physical properties of the lipid. These are thought to be present in many tissues and possibly concerned with protein synthesis. Since there is no protein synthesis in the red cell, they must either have another function or be artefacts produced during the extraction and partial breakdown of the proteolipids of the membrane.

CHAPTER 7

Discussion and Conclusions

It is now generally agreed that the transfer of glucose across the human red cell membrane is not a process of simple diffusion. A facilitated transfer has been postulated in which the essential first step is a reaction between glucose and a component of the cell membrane (LeFevre & LeFevre, 1952; Widdas, 1952). There is also a facilitated transfer of glucose in the foetal red cells of several species, for instance the sheep and guinea-pig, but after birth the rate of glucose transfer drops until its kinetics cannot be studied (Widdas, 1953b). It is unlikely that the facilitated transfer mechanism is restricted to red cells, and it is probably the first and possibly the rate-limiting step of active glucose transport in the kidney, gut and placenta.

The nature of the cell membrane component with which glucose must react is unknown, except that it must be mobile within the membrane (Widdas, 1952; Rosenberg & Wilbrandt, 1957). The nature of the reaction is also unknown, and the kinetics of transfer allow that it could be either enzyme-catalysed or a non-catalysed complex formation. In either case both association and dissociation must be very rapid, since the movement of the complex across the membrane is thought to be the rate-limiting step.

The work described in this thesis aimed to attack the problem on several fronts. An attempt to correlate the rate of glucose transfer with acid phosphatase activity in the human and maternal and foetal guinea-pig red cell has already been discussed in chapter 4, and the conclusion was drawn that no correlation exists. However, the techniques developed during this work, under the initial guidance of Dr. A.M. Bond, gave good, consistent results, and previously unreported values for

phosphatase activity were obtained for the foetal guinea-pig red cell and for the effect upon red cell phosphatases of substances known to inhibit glucose transfer. The histochemistry of alkaline phosphatase in the kidney had already been widely explored in the past few years, but acid phosphatase had been almost completely neglected and no similar studies to those reported in chapter 3 have been found in the literature. The histochemistry of the placenta sections was also interesting in showing the extremely high activity of alkaline phosphatase in that tissue.

If time had permitted, an investigation of red cell ATPase and its distribution amongst various species might have been valuable since interesting correlations have been shown to exist between the inhibitory effects of phloretin and phlorrhizin on ATPase activity and on glucose transfer (Laris, Novinger & Calaprice, 1960). These workers thought that the possibility existed that glucose transfer, ATPase activity, and thereby sodium transport, are linked. This cannot be a functional linkage since the hydrolysis of ATP does not seem to be essential for glucose transfer, but it could be a structural linkage, for instance by the sharing of certain common structures. However, work on partially disintegrated red cell ghosts suggests that the sites of ATPase activity and the sites at which the inhibitors of glucose transfer act can be separated (Ewers, Haskell & Fineberg, 1963).

As a result of the work on phosphatases the possibility could not be ruled out completely that the reaction between glucose and the membrane component was enzyme-catalysed, but nevertheless it was felt that a study of possible complexes between glucose and lipid components of membranes would be more fruitful.

When the work on the extraction of phospholipids from red cells and placental tissue was initiated, the literature was not so voluminous or informative as it is now and the techniques applicable to the problem in hand were being developed.

The early experiments were thus necessary in the development of the techniques finally used; they were also valuable in demonstrating the apparent superiority of n-butanol over the conventional chloroform-methanol as an extraction solvent. n-Butanol appears to have two properties which made it particularly suitable for extracting a glucose-lipid complex. First, its apparent ability to extract phospholipids which were associated with protein, either by releasing lipid from membrane-bound lipoproteins or by solubilising proteo-lipids. Second, the slight mutual solubility between butanol and water prevented the extraction mixtures from separating into aqueous and non-aqueous phases. From the rapid kinetics of glucose transfer it may be predicted that the hypothetical lipid-glucose complex would not be stable enough to withstand the separation of the phases which occurred in the chloroform-methanol extraction mixtures: probably much of the material of real interest in the chloroform-methanol extracts tended to accumulate amongst the interfacial material between the chloroform and aqueous phases, which was difficult to separate.

The extraction of sheep placental tissue described in chapter 5 was carried out with chloroform-methanol, and the recovery of radioactivity was rather disappointing. It might have been valuable, had time permitted, to have repeated the experiments with n-butanol as the extraction solvent. The recovery of phospholipid was fairly satisfactory, and the results showed that the lipid constituents of the sheep placenta are similar to those of human placenta, as described by Hosoya & Fujimori (1964). Phosphatidylethanolamine and phosphatidylcholine are important constituents, as in the goat (Neil, 1961), but there are substantial amount of phosphatidylinositol and sphingomyelin present as well as smaller amounts of phosphatidylserine, lysophosphatides and fast-moving substances of the polyglycerophosphatide type.

In the 3-hour placental experiments more [^{14}C] was taken up than would have been predicted from the solubility of glucose in chloroform, but it was not known what proportion of the [^{14}C] had become metabolically incorporated into the lipids. In the short-term experiments where [^{14}C] glucose or fructose was added to the placental homogenate for 20 or 60 seconds before extracting, more [^{14}C] was taken up than would be predicted from the solubility of glucose in chloroform, but possibly not more than from its general solubility in lipid.

In red cells, the differences observed between the phospholipids of sheep and humans were as predicted from the results of other workers, the most obvious features being higher cholesterol and lower lecithin in the sheep. The results with [^{14}C] glucose showed that in the human red cells cephalin fractions were slightly larger and carried considerably more [^{14}C]

than in the sheep. The radioactivity in these fractions was high enough to be located on a thin-layer chromatogram, and to be seen to coincide with a phospholipid spot.

In isolating a glucose-lipid complex by the methods described in this thesis, certain difficulties occurred. First, glucose is slightly solubilised by all phospholipids; second, the hypothetical complex between glucose and the membrane carrier component is presumably highly reversible and therefore unstable. These two factors make it difficult to distinguish between [^{14}C] due to solubilised glucose and [^{14}C] due to the proportion of glucose-carrier complex which was still associated at the moment of extraction. Another hazard is the metabolic incorporation of glucose into the lipids, but for the short incubation times being used this was probably negligible.

The inhibitor studies were carried out in the hope of obviating these difficulties. Phloretin, although a specific and strong inhibitor of glucose transfer, was difficult to detect in small amounts and its attachment to the carrier site is presumed to be reversible. The experiments with DNFB were very much more successful since it acts irreversibly at the carrier site and is obtainable as [^{14}C]-DNFB, which is easily detected.

The method used for simultaneously measuring uptake of DNFB by human red cells and inhibition of glucose transfer was valuable in that it showed the irreversibility of the inhibition and the variation of time-course with temperature. The results also demonstrated that uptake of DNFB, when plotted against percentage inhibition, is irrespective of temperature, and that this curve may be used to calculate the number of DNFB molecules

required per red cell to fully inhibit the glucose transfer system. The value obtained - 400 million molecules per red cell - was obviously an over-estimate of the number of carrier sites since it must include a large proportion which was taken up by haemoglobin and glutathione. Extraction with n-butanol of the DNFB-inhibited red cells and separation of the lipids gave a yield of DNFB in the lipids of 10-20 million molecules of DNFB per red cell. This comfortably exceeds the total amount of DNFB which would be necessary to give total inhibition if one accepts the estimates of the number of carrier sites of other workers - 500,000 per red cell ghost (LeFevre, 1961) and 700,000 per red cell (Weed, van Steveninck & Rothstein, 1964). Of the [^{14}C] DNFB recovered in the lipid fractions from silicic acid column chromatography, 20% was in the cephalin fractions, and this alone would more than account for the sites. Some of the material eluted in chloroform-methanol 1:4 has a high R.A./P ratio, and probably contains lysoderivatives of the cephalins.

If the amount of [^{14}C] eluted in the various fractions of the sheep lipids is compared with the human (see Table XXIV), it may be seen that the total uptake of [^{14}C] in the cephalins and lyso-cephalins is 2,230 counts/min. for the sheep and 2,980 counts/min. for the human. That is, the human exceeds the sheep by 750 counts/min., which is 10% of the total count, and this represents one million molecules DNFB per red cell. This again exceeds the estimated number of sites.

It cannot be assumed that because DNFB reacts with phospholipids of the cephalin group, cephalins are necessarily the membrane carrier component, since DNFB may exert its inhibitory effect by attacking groups

in the membrane adjacent to the carrier sites. Nevertheless, a comparison between elution curves obtained in the [^{14}C] glucose and [^{14}C] DNFB experiments does demonstrate that in each case only the cephalins and lyso-cephalins have a high R.A./P ratio which cannot be ascribed to the presence of free [^{14}C] glucose or [^{14}C] DNFB. In each case, the radioactivity was shown to be associated with a phospholipid spot on thin-layer chromatography, and Miss Mary Forsling was able to detect amino-groups on hydrolysis of the spot, although not with any regularity.

The bulk of the radioactivity in the cephalin fractions was associated with the phosphatidylethanolamine spot, but, since this spot on hydrolysis did not necessarily yield ethanolamine and sometimes yielded a variety of other amino-acids, it is impossible to conclude that phosphatidylethanolamine is the mobile membrane component. The most satisfactory working hypothesis would be that a variety of lipo-amino-acids are extracted from red cell membranes by n-butanol and are subsequently eluted on the silicic acid columns in the cephalin fractions. Such lipo-amino-acids have been characterised by Macfarlane (1962) as o-amino-acid esters of phosphatidylglycerol, and have been reported in hen oviduct mince, liver slices and ascites tumour cells. Wren (1960) considered the possibility of their being artefacts arising from binding of nitrogenous substances during lipid autoxidation, but concluded that at least some of them are genuine entities, for instance phosphatidylserine which has been isolated pure and its chemical structure established. It is hoped eventually it will be possible to isolate a sufficient amount of the glucose or DNFB-complexed lipid to identify it with confidence. Even then it will not

follow that an association between glucose and the amino-group of a particular phospholipid is necessarily concerned with glucose transfer. It is known, for instance, that the carbonyl groups of sugars readily form linkages with the free amino-groups of proteins, particularly with lysine and methionine (Lea & Hannan, 1950); they showed this to occur in slightly moist casein-glucose mixtures to such an extent that the food value of dried milk is severely affected.

It was not found possible to isolate separate phospholipids from the red cell extracts in sufficient quantity to study their individual behaviour with glucose. If it had been possible, monolayer studies of the type described in chapter 5 might have been valuable. The preliminary work on monolayers, using the standard phospholipids then available, measuring changes in surface pressure and surface potential on compression in the absence and presence of glucose, showed it to be a sensitive technique and probably capable of fair reproducibility. In the experiments described in chapter 5, insulin was added to the monolayers as a stabiliser since it is a protein of low molecular weight containing a wide variety of amino acids. The results suggest that cholesterol may have been more satisfactory. When insulin was chosen it was assumed to have no effect on glucose transfer in the red cell. Recent work by Rieser and Rieser (1964) has shown that insulin may, in fact, have an accelerating effect on glucose uptake if the red cell is first treated with chrymotrypsin, presumably to remove a surface layer of protein overlying the lipid components of the membrane.

With the purer phospholipids now available and with improved techniques for obtaining separate phospholipids from red cell extracts, monolayer studies may have a useful place in the analysis of interactions between lipids and glucose.

SUMMARY

1. A review has been made of the properties of cell membranes, especially red cell membranes, with particular reference to glucose transport. The phosphorylation theory of glucose transport has been discussed. The phospholipids found in cell membranes have been described and their properties discussed, and the possibility also discussed that the hypothetical membrane carrier component in facilitated glucose transfer is a phospholipid.

2. The methods have been described for the estimation of phosphatase activity. The results for maternal and foetal guinea-pig red cells ~~and kidney~~, and the effect upon their phosphatase activity of inhibitors known to affect glucose transfer, have been presented, and the conclusion drawn that no correlation exists between phosphatase activity and glucose transfer in the red cell. Histochemical studies were presented to show the distribution of acid and alkaline phosphatase in the guinea-pig kidney and placenta.

3. The methods have been described which were developed to extract from sheep placental tissue and from red cell membranes the hypothetical lipid-glucose complex. The superiority of n-butanol as an extraction solvent was demonstrated.

4. The phospholipids present in sheep placental tissue have been described, and their uptake of [^{14}C] glucose and [^{14}C] fructose in both long-term and short-term experiments discussed.

5. The phospholipids present in sheep and human red cell lipids have been described, and their uptake of [^{14}C] glucose in short-term experiments.

6. The uptake of [^{14}C] DNFB by human red cells has been measured, and calculated to be 400 million molecules per red cell to give full inhibition of glucose transfer. This is assumed to be a great over-estimate of the number of carrier sites present in the red cell membrane.

7. Lipids from [^{14}C] DNFB-inhibited red cells were extracted and separated, and radioactivity found to be in the cephalin fractions. The possibility has been discussed that phosphatidylethanolamine is the membrane carrier component, but no conclusion has been drawn.

8. The possibilities have been explored of using monolayer studies to examine the effect of glucose on pure phospholipids.

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Uptake of dinitrofluorobenzene by human erythrocytes

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It was shown by Bowyer & Widdas (1956) that 1-fluoro-2-4-dinitrobenzene (DNFB) was an irreversible inhibitor of facilitated glucose transfer in erythrocytes. When red cells were incubated with DNFB (1·4 mM) at 21° C, inhibition developed rapidly at first but took up to 4 hr to reach 99 % inhibition.

In the present experiments, the uptake of inhibitor has been measured using ^{14}C-labelled DNFB and parallel determinations of the inhibition of glucose transfer have been made. The uptake of radioactivity had a rapid initial phase followed by a more gradual rise, reaching about 2×10^9 molecules per erythrocyte when glucose transfer was 99 % inhibited.

By incubating with 1·4 mM DNFB at temperatures between 15° and 33° C, the time course for development of inhibition varied sixfold and the time course for uptake of radioactive DNFB was similarly affected. The relationship between inhibition and uptake was thus almost independent of the temperature of incubation. From the steepest part of the inhibition–uptake curve, it can be estimated that full inhibition would require a minimum uptake of 4×10^8 molecules per cell.

When butanol extracts of inhibited cells were fractionated it was found that labelling was present in some lipid fractions, but this represented less than 1 % of the DNFB taken up by the cells. That these lipids might ordinarily be capable of complexing with glucose is suggested by the observations of LeFevre, Habich, Hess & Hudson (1964) that the phospholipids of cells inhibited by DNFB are less able to promote the solubilization of glucose into hexane. The labelling appeared in fractions which were eluted from a silicic acid column by chloroform–methanol mixtures (9:1) and (1:4). The concentration of these compounds was of the order 4×10^6 molecules per cell and 3×10^6 molecules per cell respectively.

Stein (1964) has recently made a study of the peptides in erythrocyte ghosts which take up labelling when the erythrocytes are treated with radioactive DNFB at pH 6 and in 10 % alcohol. Peptide labelling in his experiments was of the order of 2×10^6 molecules per red cell ghost.

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