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THE EFFECTS OF INSULIN, INSULIN DERIVATIVES AND ORGANIC MERCURIALS ON THE EXCHANGE AND MAXIMAL NET COMPONENTS OF GLUCOSE EFFLUX IN THE HUMAN ERYTHROCYTE

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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INTRODUCTION

Transport

The precise manner in which glucose and other non-electrolytes penetrate the membrane of the human erythrocyte is as yet unknown. Observations obtained primarily from kinetic and inhibitor studies have led to a generalized theory of facilitated transfer to explain the movement of glucose across the membrane of human red cells. Facilitated transfer is believed to operate by means of a temporary, stoichiometric association of the substrate molecule, glucose, with a limited number of reactive sites present in or on the cell surface. These reactive sites are referred to as 'carriers'. Because of its size and hydrophilic nature, it would be expected that glucose would enter the red cell very slowly if passive diffusion were solely responsible for its entry. However, studies of the permeability of the human red cell to glucose have shown that this molecule enters and leaves the human erythrocyte in a manner (i.e., a much faster rate at low concentrations and a much slower rate at high concentrations) not predicted by the laws of simple diffusion.

Kozawa ('14) described the permeability of the human red cell to hexoses and pentoses. His report that isomeric sugars penetrated the red cell at different rates suggested that a rather refined structural specificity was involved. Kozawa ('14) also noted large differences in permeability between species. Sugar penetration into human and macaque red blood cells took place rapidly while permeability into the red blood cells of a variety of adult rodents, carnivores and ungulates was considerably slower, almost to the extent that they have at times been considered as sugar-impermeable.

The permeability of the human red cell to polyhydric alcohols and sugars was further studied by Jacobs ('34) whose quantitative treatment

of the results obtained allowed for the formulation of permeability constants. Bang and Ørskov ('37) found that on raising the concentration of glucose from 20 to 50mM in a suspension of red cells and saline, the permeability constant was reduced 2.5 times.

The transfer of non-electrolytes across the red cell membrane in these cases was measured optically by recording changes in light transmission through very dilute suspensions of erythrocytes. This method (Ørskov, '35 and Parpart, '35) is based on an empirical relation between the average red cell volume in the sample and the fraction of light which is scattered in passing through the suspension. By adjusting the concentration of the cells in suspension and arranging instrument factors, a linear relationship can be established between changes in scattering and changes in cell volume. Since water equilibrates through the red cell membrane much faster than any specific solute, the osmotic water movement may be considered instantaneous without the introduction of any appreciable error. Thus, the cells may be considered to be in osmotic equilibrium with the suspension medium and volume changes with time serve as a measurement of the transfer of a sugar in osmotically equivalent amounts. Though indirect, the linear correlation of the recorded deflections due to scattering with osmotic alterations in the cell volume has been well corroborated. Similar measurements on glucose permeability were made by Wilbrandt ('38, '50) using the photometric method developed by Parpart ('35) which is based on the continuous recording of the increase in optical transmission accompanying hemolysis. This method involves following either: 1. The course of hemolysis as sugar enters the cells from a medium in which the salt concentration alone is insufficient to maintain cellular integrity, or 2. The progressive changes in the cells' osmotic resistance curve after addition of sugar to an isotonic medium.

These several early observations lay relatively dormant until LeFevre ('48) began a series of investigations into the permeability of the red cell membrane to glucose and glycerol. It was shown that glycerol transfer (LeFevre, '48) is blocked by copper, mercuric chloride, p-chloromercuribenzoate, iodine and phloridzin and that glucose permeability is similarly depressed by mercuric chloride, p-chloromercuribenzoate, iodine and phloridzin but is not affected by copper. In addition, LeFevre ('48) suggested that, as a result of the kinetic data obtained from volume changes in various glucose-saline solutions, it appeared that the mechanism for the transport of glucose into the cell was regulated by the existing intracellular concentration rather than by simple diffusion gradients. The kinetics of glucose transfer were further investigated and the results so obtained provide the primary evidence for the operation of facilitated transfer. Of particular relevance is the fact that the kinetics of glucose efflux show a saturation effect. It was possible to demonstrate that at any given concentration gradient between cells and environment, the rate of glucose entry will decrease at higher outside concentration and that the equation describing the penetration of sugar into sugar-free cells should be comparable to the Langmuir adsorption isotherm or the Michaelis-Menten equations. Using the Langmuir adsorption isotherm, Widdas ('54) presented the following equation for calculating the rate of sugar entry:

$$(1) \quad \text{transfer rate} = K\left[\frac{Co}{Co + \emptyset} - \frac{Ci}{Ci + \emptyset}\right]$$

where C_o refers to the concentration of sugar outside the cell, C_i to the concentration of sugar inside the cell, K is a proportionality constant and Ø denotes an equilibrium constant for a carrier-hexose complex. When the reactive site is not saturated, e.g., if the sugar has a low affinity

for the site or is used in low concentration, and $\emptyset >> C_o$, C_i, equation I reduces to:

$$(2) \quad \text{transfer rate} = \frac{K}{\emptyset}(C_o - C_i)$$

under these conditions, the rate of transport will be proportional to the reciprocal of Ø and directly proportional to the difference in the sugar concentrations inside and outside of the cell. The kinetics depicting such a condition will not differ significantly from those of simple diffusion and have been termed D-kinetics (Wilbrandt, '54). It has been demonstrated that D-kinetics hold for sorbose and fructose transfer in human erythrocytes as well as for glucose at very low sugar concentrations e.g., concentrations where the reactive site is well below saturation.

At high concentrations where the reactive site is saturated and where C_i and $C_o >>> \emptyset$ the rate of sugar transport is described by equation 3:

$$(3) \quad \text{transfer rate} = K\emptyset\left[\frac{1}{C_i} - \frac{1}{C_o}\right]$$

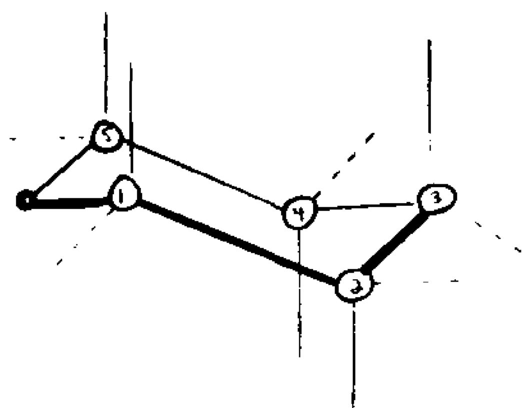
Here, the rate of transfer is proportional to the difference in the reciprocals of the sugar concentration inside and outside the red cell and directly proportional to Ø. These kinetics have been termed E-kinetics (Wilbrandt, '54) and have been shown by several investigations (Widdas, '54; Wilbrandt, and Rosenberg, '51; Wilbrandt et al., '56 and LeFevre and Davies, '54) to describe accurately the penetration into the human erythrocyte of those sugars which have a high affinity for the reactive site.

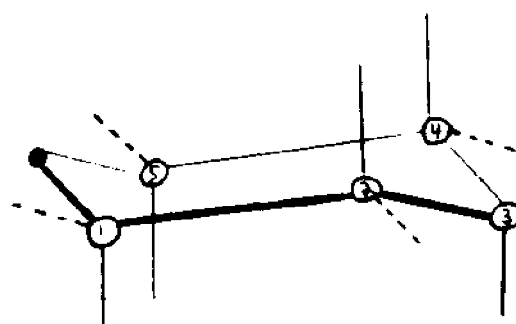
In addition to the kinetic data, various investigations utilizing different inhibitors have been made on glucose transport. Glucose transfer has been shown to be inhibited by mercuric chloride, p-chlormercuribenzoate, iodine, phloridzin (LeFevre, '48); phloretin, polyphloretin, the lachrymators allyl isothiocyanate, bromacetophenone, and chloropicrin (Wilbrandt, '54); and dinitrofluorobenzene, dinitrochlorobenzene and

dinitrobromobenzene (Bowyer, '54 and Bowyer and Widdas, '56). The inability of a number of metabolic inhibitors, fluoride and cyanide (Kozawa, '14), iodoacetate (LeFevre, '48), 2,4-dinitrophenol and azide (Bowyer, '54) to affect glucose transfer in the red cell has indicated this transfer to lack any active component. The ability of mercuric chloride and organic mercurial derivatives (LeFevre, '48) to inhibit glucose transfer as well as the almost complete reversal (LeFevre and Davies, '51 and LeFevre, '54) of the inhibitory action upon the addition of cysteine focused attention on the sulphydryl groups at the cell surface. The ability of the fluorobenzene derivatives (Bowyer and Widdas, '56) to effectively inhibit glucose transfer in human erythrocytes pointed toward an involvement of a protein component in the cell surface.

The stereospecificity of glucose transfer is another aspect of this phenomenon which has been investigated. The results so obtained have lent significant support to the concept of facilitation. The report of Kozawa ('14) on differences in the permeabilities to isomeric sugars was confirmed and extended by Wilbrandt ('38,'47) who showed that differences between direct enantiomorphs occur. D-xylose and L-arabinose enter the red cell rapidly while L-xylose and D-arabinose appear to be completely excluded. In addition it was reported that D-xylose and L-arabinose interfered with each other during simultaneous entry. It was also shown that stereoisomers (D-glucose, D-mannose, D-galactose, L-sorbose and D-fructose) penetrate at different rates and that in addition optical isomers (D- and L-glucose) show different entry rates. When the penetration rates of mixtures of non-electrolytes are examined, competition between the various sugars is observed. It was found that D-glucose, D-galactose, D-mannose, L-sorbose, D-fructose, D-xylose and L-arabinose all compete with one another in their movement across the red cell membrane (LeFevre and Davies, '51).

The importance of steric factors in transport phenomena resulted in an interesting investigation by LeFevre and Marshall ('58). They compared fourteen different sugars with respect to their affinity for the transport systems and found that affinity was directly related to the stability of the pyranose ring in the C I chair form. The C I conformations of D-glucose, D-mannose, D-galactose, and D-xylose, sugars with a relatively high affinity for the human red blood cell transport system, are many times more stable than the IC conformations of those sugars. Figure I illustrates the two possible chair conformations of the pyranose ring. In the C I conformation of the above sugars, the carbinol group as well as most of the hydroxyl groups are in the equatorial position in the plane of the ring, while the hydrogen atoms are in the axial position, perpendicular to the ring. In the I C conformation, however, the carbinol group of D-glucose, D-mannose, and D-galactose as well as at least two hydroxyl groups of all four hexoses are in the axial position, an arrangement of greater instability. Since there would be more interference among axial than equatorial groups, it would appear that the conformation in which the smaller groups are in the axial position should be favored. Indeed, this is borne out by the results which show that the sugars which are most stable in the C I conformation have the highest affinity for the red blood cell transport system, while those sugars which exhibit greater stability in the I C conformation (L-glucose, L-galactose, L-xylose) show extremely low affinities. Because of the nature of this discrimination between CI and I C conformations, it was concluded (LeFevre, '61) that the reactive site on the red cell surface must be able to distinguish between the right and left handed forms of the sugar molecule. It was also felt that such a refinement would require a three point contact between molecule and membrane component, for only such a contact at the minimum would be sufficient to distinguish between the two





CONFORMATION C I

CONFORMATION I C

FIGURE I. Chair conformation of the pyranose ring. Filled circle represents the O - atom; numbered circles represent the carbons as conventionally numbered in aldoses. Heavy lines mark the side of the ring facing the observer. "Equatorial" extracyclic bonds are shown by broken lines; "axial" bonds by vertical solid lines. (LeFevre and Marshall, '59)

chair forms of the pyranose ring.

From the study of the kinetics of transport and with the aid of inhibitor and sugar specificity investigations, several additional characteristic features were recognized: 1. It could be shown that even though there is no uphill transport of glucose in the red cell, an apparent uphill transport can be demonstrated under certain conditions. Thus, the phenomenon of "counter-transport" (Rosenberg and Wilbrandt, '57) is observed - that is a linkage between the facilitated movement of a substance down its electrochemical gradient with the movement in the opposite direction of a structurally analogous molecule, 2. Glucose enters the red cells of primates approximately 10^4 times faster than could be accounted for on the basis of simple diffusion through a lipid layer (Jacobs, '34 and Jacobs and Parpart, '33), 3. The temperature coefficient for glucose transport in human red cells is 2.5 (Bowyer, '54), 4. Definite species differences for glucose and glycerol penetration have been observed. Widdas ('55) has shown in this respect, that erythrocytes from fetal blood of the pig, rabbit, guinea pig, sheep and deer resemble those from adult human blood, all possessing a high glucose permability. This high level begins to diminish at the time of birth, and 5. There may also appear clear differences between the rate of penetration of glucose when the net transfer is measured in contrast to the unidirectional flux of isotopically labeled permeant. It has been reported that the latter flux is significantly greater than the net flux (Britton, '56; LeFevre and McGinniss, '60); Levine et al., '65; and Mawe and Hempling, '65).

As a result of the data gathered from kinetic, inhibitor and other studies, a number of proposals depicting models for facilitated transport have been suggested. Several of these (LeFevre, '48; LeFevre and LeFevre, '52; Widdas, '52 and Bowyer, '57) postulated the presence of a carrier

which is in or on the cell surface. The carrier, accordingly, reacts with a sugar molecule at one surface in order to form a sugar-carrier complex which can move across the membrane by ordinary thermal agitation in the direction of its concentration gradient. At the other surface the carrier releases the sugar molecule. A similar theory (Rosenberg and Wilbrandt, '55), has been proposed with the modification that the formation and dissociation of the complex is catalyzed by an enzyme or enzymes in the membrane. The passage of sugar through a protein-lined pore has been suggested by Danielli ('54). In this model, the sugar would traverse the membrane by becoming attached to one site after another. It is essentially similar to a polar "creep" hypothesis put forth by Bowyer and Widdas ('56). The observations however that counter-transport (Rosenberg and Wilbrandt, '57) could be induced in human erythrocytes tended to overrule, though not completely, the concept of transport via a pore. It was shown that when mannose was present in equal concentrations inside and outside the human red cell, additional mannose would leave the cell when glucose was added to the external medium. This temporary movement up a concentration gradient cannot be too readily explained in terms of a non-mobile membrane component. A more or less general consensus for a model of facilitated transfer via a mobile carrier is depicted in Figure 2 (LeFevre and McGinniss, '60). In this model the rate-limiting step is considered to be the movement of the carrier-permeant complex through the membrane.

Kinetics which were previously presented are based on the assumption that the free carrier and the carrier-permeant complex travel at the same rate across the membrane. Evidence that this is not necessarily the case has been recently presented. Britton ('56) has shown that the flux of C-14-labeled glucose under equilibrium conditions differed from the flux values obtained when a net movement of the permeant is measured.

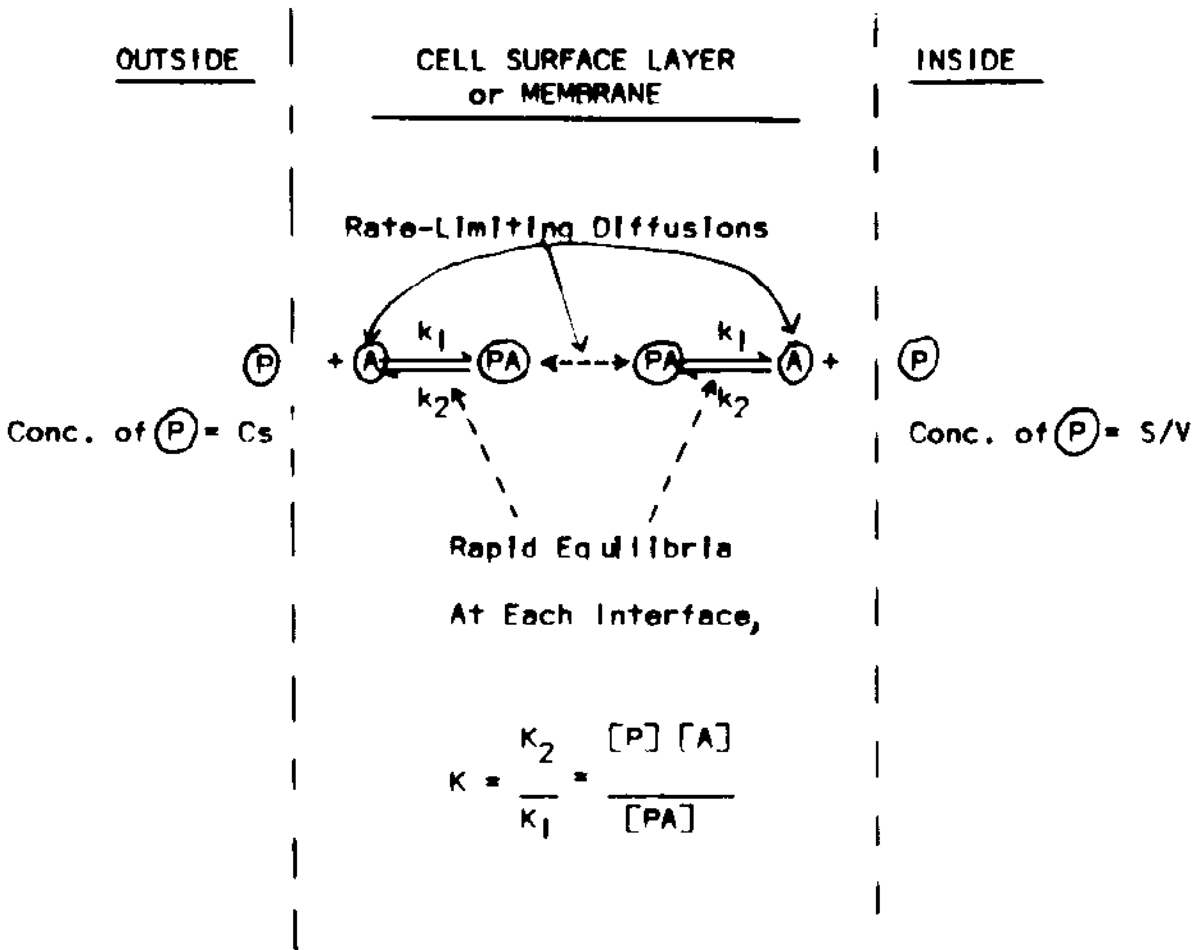


FIGURE 2. Model of facilitated diffusion system. The penetrant P as such is unable to enter the cell membrane, but it combines reversibly with carrier A at either interface, rapidly approaching equilibrium defined by dissociation constant K. The slower diffusion of complex PA through the membrane, according to its concentration gradient within the membrane, determines the over-all migration of P. (LeFevre and McGinniss, '60)

For the sake of clarity these terms, equilibrium, exchange and net flux will be defined now as follows: equilibrium flux is the same as exchange flux and is the measurement of either the influx or the efflux of glucose when the concentrations of the sugar inside and outside the red cell are equal during the course of an experiment. By obvious necessity this is an isotopic measurement and is also termed the unidirectional flux of the tracer. The net flux is a measurement of the change in glucose content of the red cell when there is a difference between the internal and the external concentrations of the sugar. The additional term, maximal net flux, will also appear and this is the measurement of the net flux under ideal conditions, that is, when the concentration of the sugar on one side of the membrane remains well above the saturation level of the reactive site while on the other side the concentration of the sugar remains as close to zero as possible for the length of the measurement. Wilbrandt ('61) has shown that true maximal net fluxes will be measured when precautions (such as the use of a large compartment into which the glucose can move) are taken to prevent back flux. LeFevre and McGinniss ('60) measuring glucose influx reported the equilibrium flux to exceed the net flux by fifty to one-hundred times. In another investigation, Mawe and Hempling ('65) measured glucose efflux over a wide concentration range and reported the rate at which glucose left the cell under equilibrium condition was only two and one half times faster than the rate under maximal net flux conditions. These results were confirmed independently by Levine et al., ('65). The difference in the results between these two reports is attributed to the fact that the measurements of net glucose influx by LeFevre and McGinniss were not really measurements of maximal net flux. As described previously, the

concentration of the permeant should remain very close to zero or by the same token well below the saturation level of the carrier in order to prevent back flux. Such a situation is readily attainable in efflux studies. However, in measurements of influx, the volume of the red cell being relatively small, the concentration of the permeant rises rapidly and may easily approach the Km value (reported as between one and two millimoles by Sen and Widdas, '62) of the carrier. When this occurs the conditions for measuring maximal net flux are no longer present.

This difference between equilibrium and maximal net flux provides further support for the mobile carrier theory. Utilizing a series of expressions by Britton ('64), and substituting the experimentally determined value of 2.5, the ratio of the equilibrium flux to the maximal net efflux, Mawe and Hempling ('65) calculated that the carrier-permeant complex may be shown to cross the membrane four times faster than non-complexed carrier.

In summary, as a result of these numerous investigations, the carrier is generally considered as a membrane component which is characterized by the following: 1) The carrier is present in the cell membrane in limited amounts and its combination with permeant occurs by as yet undefined bonds, 2) The carrier can combine with the permeant to form a complex of variable stability, 3) The extent of this combination is dependent on the nature of both carrier and permeant, 4) The complex has the property of translocation, 5) The permeant transfer across the membrane is negligible when not combined with carrier, and 6) The rate of translocation of uncombined and complexed carrier can be different.

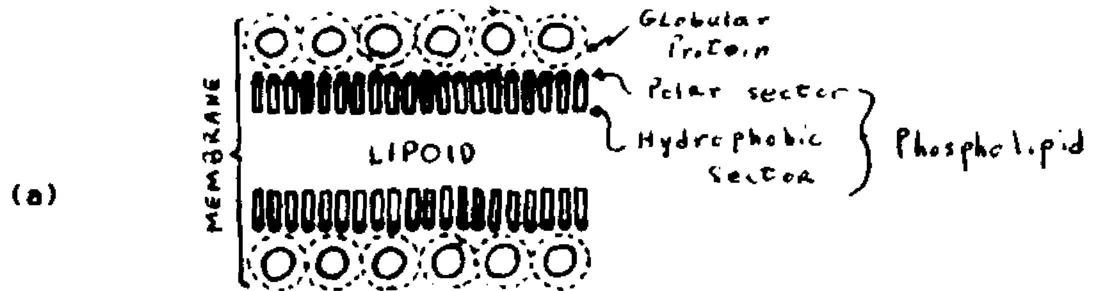
Membrane

The membrane, from all of the evidence gathered so far, appears to be the repository of the carrier sites. Thus, the membrane and its composition and structure, are of particular significance in the investigation of transport phenomena. The Danielli-Davson paucimolecular model (Danielli and Davson, '35) of the ultrastructure of the plasma membrane put forth to explain the permeability of cells is shown in Figure 3a. The features of this model are: 1. One or more bimolecular leaflets of phospholipid are sandwiched between two layers of protein considered to be globular, and 2. Lipid and protein constitute essentially separate but continuous phases with the polar groups of the phospholipid being bonded electrostatically to protein. This picture of the membrane was further refined by Robertson ('60). His model for the "unit" membrane is shown in Figure 3b. Like the Davson-Danielli model, the concept of phospholipid layers sandwiched between protein layers is retained. Similarly, both membrane models consider the bonding between phospholipid and protein to be primarily electrostatic and that the lipid and protein are essentially separate phases. The unit membrane model considers the membrane to be asymmetric rather than symmetric, with mucopolysaccharide or mucoprotein on the outside face and unconjugated protein on the inside face. The protein layers are visualized as extended polypeptide chains less than 20Å thick and the bimolecular leaflet thickness is set at about 35-40Å.

The membrane models, especially the latter, are based on the assumption that all membranes are quite similar and that myelin, the substance on which the unit membrane is based, is a representative example for all membranes.

A number of objections have been raised, however, to these assumptions. Several different investigations have indicated that: 1. Hydrophobic rather than electrostatic binding is the predominant binding between lipid

(a)



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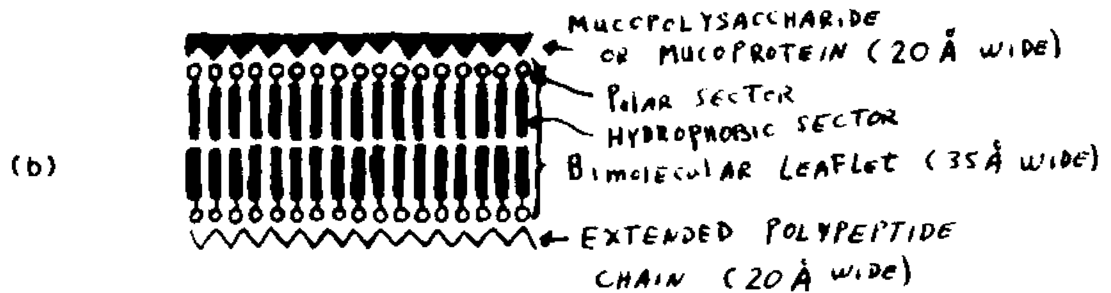


FIGURE 3. (a) Danielli-Davson model of membrane structure.
(b) The 'unit membrane' model of Robertson.

and protein (Richardson, et al., '64 and Brown, '65), 2. The binding between protein and phospholipid is very tight and they probably do not exist as separate phases (Fleischer et al., '62), 3. The protein layer of the erythrocyte membrane is not in the extended beta configuration (Maddy and Malcolm, '65; Korn, '66; Lenard and Singer, '66; Hoelzl Wallach and Zahler, '66 and Blackwell et al., '65) but is randomly coiled (15% alpha helix), and 4. The complexes which can form as a result of combinations of various lipid components can lead to micellar and pore formation (Lucy and Glauert, '64).

The role that lipids can play in the structure of the membrane with respect to such aspects as lipid content, composition and the nature of the acyl moiety has been discussed by O'Brien ('67). The compactness of the structure can be shown to vary considerably depending on these variables alone and the variations in these items among myelin, red cells, mitochondria and chloroplasts are considerable. On the basis of the lipid content alone (de Gier and van Deenen, '61; Nelson, '67; and Nelson, '67a) it is possible for the red cell membrane to possess areas (patches) quite dissimilar from that visualized by the "unit" membrane model. The difference in the degree of compactness of the membrane structure can be examined for red cells of different species. When the parameter is stability to glycerol hemolysis, it has been shown (Jacobs et al., '36 and Jacobs et al., '37) that ox erythrocytes are more stable and that susceptibility to hemolysis increases with human, rabbit and rat erythrocytes in that order. The lipids of the erythrocytes (Parpart and Dziemian, '40 and Parpart and Ballentine, '52) of these species differ with regard to content of long chain sphingolipids (primarily sphingomyelin). The ox red cell contains very high levels of sphingomyelin and very low levels of polyunsaturated phosphatides while at the other end of the scale the rat erythrocyte con-

contains low levels of sphingomyelin and high levels of polyunsaturated glycerophosphatides. It can be demonstrated that the more condensed bilayer configuration will occur when the level of sphingolipids is high and the polyunsaturated lipid level is correspondingly low. Thus, the greater stability of ox erythrocytes to glycerol permeability and hemolysis may be attributed to the lipid makeup of the bilayer configuration. Whether this is so for this particular case is not as important as the evidence which demonstrates the vast possibilities for diversity from membrane to membrane and from one area of the same membrane to an adjacent area.

With this in mind, a closer look at the erythrocyte membrane reveals some interesting findings which may be of use in understanding sugar transport. The determination of the ultrastructure of the red cell has proven to be a most interesting and difficult task. The newest techniques of thin sectioning and freeze etching have greatly added to these studies. The red cell interior contains a high concentration of hemoglobin in a quasicrystalline state. There is still however, no clear cut evidence for the existence of a supporting protein network in the interior of the red cell. As to the inside peripheral layers, it appears from the work of several investigators (Hoffman, '58 and Dodge et al., '63) that hemoglobin is neither tightly bound to the inner side of the membrane nor does it form a structurally fixed component.

The plasma membrane forms a boundary layer between two compartments and may be defined operationally as that part of the cell which regulates permeability. By means of replication (a technique for reproducing surface textures) and shadow casting, it can be shown (Hillier and Hoffman, '53) that there exists over the surface an array of randomly placed plaques, 30Å thick and 100-500Å in diameter.

The plaques can be removed from the surface by ether but are themselves unaffected, thus leading to the conclusion that the plaques are attached to lipid in the membrane. In other preparations which were not shadowed it was possible to see fibers, 20Å in diameter and with an average length of 200Å, arranged tangentially to the cell surface. The fine structure of the fibers are unaltered by ether and it is assumed that they are bound to lipid.

When the red cell is properly prepared and thin sectioned, electron micrographs show a rather consistent picture of the cell membrane as a three-layered complex, made up of two dark lines, each 25A thick, separated by a less dense interspace of 20Å. This complex is typical of the Robertson unit membrane (Robertson, '60) and agrees with the Davson-Danielli (Danielli and Davson, '35 and Danielli, '36) model of the membrane as a bimolecular leaflet of lipid with protein at both outer surfaces. Due to uncertainties (Kinsky et al., '67) arising from the effects of the various fixing methods, it is difficult to interpret this three-layered complex. In addition, a completely lamellar structure could not be consistent with the functional qualities inherent in the membrane. A model which incorporated some micellar structure, even if only transient and slight in nature, would allow for ion permeability. A model of the red cell membrane proposed by Whittam ('64) which attempts to incorporate the physical chemical data is shown in Figure 4. Supporting the possibility of transient micellar substructure are the experiments of Seeman ('66) who has been able to demonstrate ferritin entrance during osmotic hemolysis and as such may have demonstrated the presence of transitory pores in red cells.

Recent measurements of the surface area of the human red cell indicate it to be $145 \pm 8\mu^2$ (Westerman et al., '61) rather than the $99\mu^2$ obtained

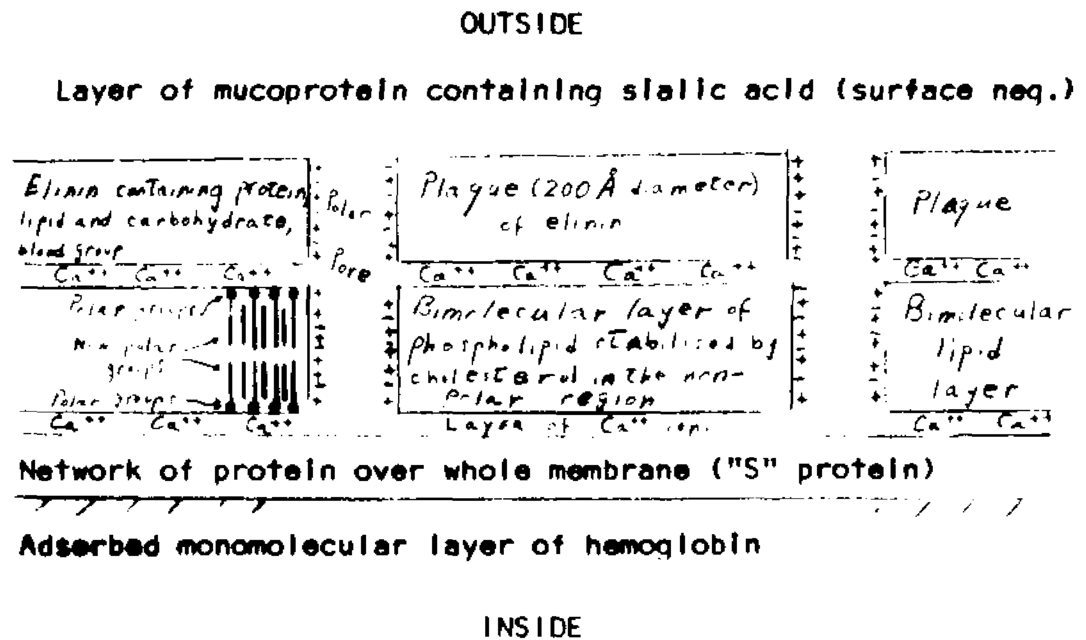


FIGURE 4. Membrane model according to Whittam. This model of the red cell membrane includes an outer mucoprotein layer, plaques, a bimolecular leaflet of phospholipid, and a layer of protein adjacent to the hemoglobin. Polar pores may be lined with protein so as to give a net positive charge.

by Gorter and Grendel ('25). Gorter and Grendel ('25), using acetone, extracted the lipids from erythrocytes, spread the extract as a monomolecular film and determined the ratio of film area to erythrocyte area. They found a ratio of 2:1 which suggested a bimolecular layer of lipids at the erythrocyte surface. This provided the basis for the Danielli-Davson membrane model (Danielli and Davson, '35). However, the accuracy of their lipid determinations has been questioned and new figures for the surface area (the one above = $145 \pm 8\mu^2$) as well as one reported by Seeman ('67) of $163 \times 10^8 Å$ would appear to make it less likely that a bimolecular lipid layer is present over the entire surface at all times.

From x-ray diffraction studies (Rand and Luzzati, '68) it can be shown that cholesterol, present in high concentrations in erythrocyte membranes ($1.39 \pm .07 \times 10^{-10}$ mg. per cell) (Bar et al., '66) is located so that part of its steroid nucleus is between the polar groups of the phospholipid molecules while the rest of the molecule extends into the inner hydrocarbon layer. As such, the presence of cholesterol causes a reduction in area occupied by phospholipid molecules. It therefore appears that the condensing effect of cholesterol observed in a monolayer of unsaturated phospholipids at an air-water interphase may also occur in the fully hydrated bimolecular layer as well.

When human red cells were treated with phospholipase C, it was reported that (Lenard and Singer, '68) up to 74% of the total membrane phosphorus was released into solution as a result of the hydrolysis of membrane phospholipids to diglycerides and water-soluble phosphorylated amines. Investigation of the state of the membrane by means of phase microscopy indicated the membrane to have remained intact. In addition, the structural protein conformation appeared unaffected. As a result of these findings and other measurements dealing with optical rotatory dispersion spectra

and circular dichroism measurements of membrane proteins, these authors postulate a membrane model which is shown in Figure 5. In this model, the authors propose that the ionic and polar heads of the phospholipids, together with the charged groups of the proteins are all situated at the exterior surface of the membrane in contact with the bulk aqueous phase. The interior of the membrane contains the hydrophobic tails of the phospholipids, the rest of the protein and other hydrophobic components such as cholesterol. This contrasts with models of the Davson-Danielli-Robertson type, where it is suggested that the ionic and polar heads of the phospholipids are submerged under a monolayer of protein on both surfaces of the membrane and that the entire structure is held together predominantly by electrostatic interactions between the ionic heads of the phospholipids and the charged groups of the protein monolayers. The experimental results (Lenard and Singer, '68) indicated that: 1. Phospho-ester bonds that are hydrolyzed are readily accessible to phospholipase C in the intact membrane, and 2. Electrostatic interactions between phospholipids and membrane proteins play only a secondary role in maintaining cell membrane integrity and in the determination of the conformation of membrane proteins.

In viewing the overall picture of non-electrolyte transport, mention should be made of certain substances (the so-called "minor" components) that are part of or are easily removed from the red cell membrane. Though in most cases evidence for direct involvement of these components with the glucose transport system is lacking, they are part of the membrane system and may prove to be necessary adjuncts in the formulation of a meaningful concept of non-electrolyte transport.

A number of these minor components of the red cell membrane can be removed by treatment of the cells with proteolytic enzymes. Sialoglyco-

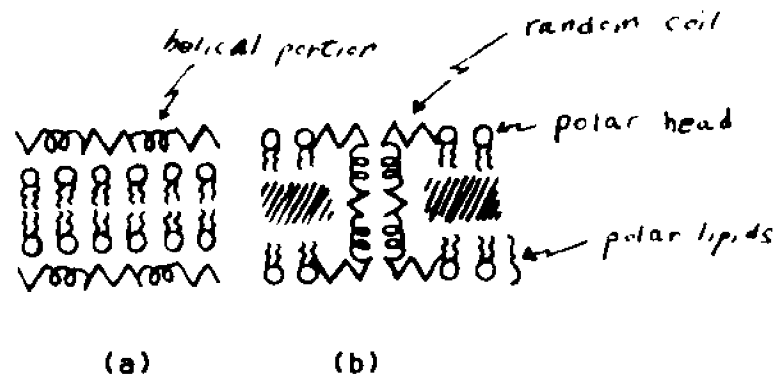


FIGURE 5. (a) The Danielli-Davson-Robertson unit membrane, as modified to include the data presented by Lenard and Singer. The proteins on the outer surfaces of the membrane consist of helical and random coil portions. The polar lipids are oriented in a bimolecular leaflet with their polar heads facing out. (b) A generalized membrane suggested by Lenard and Singer. The cross-hatched areas are assumed to be occupied by relatively nonpolar constituents (hydrophobic amino acid residues or lipids).

peptides have been released by treatment with pronase (Ohkuma and Shinohara, '67) and trypsin (Ohkuma and Ikomoto, '66, and Winzler et al., '67). These glycopeptides fall into a 10,000 molecular weight size group, contain galactose, acetyl glucosamine, acetyl galactosamine, N-acetylneuraminic acid and are rich in serine and threonine. There have not been any reports however, which demonstrate any connection between the release of these glycopeptides and the sugar transport system in human erythrocytes. Flux measurements (Odesser and Mawe, '67) following trypsin and chymotrypsin digestion have indicated these enzymes to be without any effect on the glucose transport system.

There have been suggestions that enzymes might play a role in hexose transport in the human red cell and one of the enzymes strongly considered has been mutarotase. Until recently, the presence of this enzyme had not been demonstrated in erythrocytes, though its existence there had long been suspected. The presence of the enzyme mutarotase has now been demonstrated by Sacks ('68) in lysed erythrocytes and in hemoglobin. The author presents a number of experimental behavorial similarities which raised the possibility of some role for mutarotase in the transport of glucose in erythrocytes.

Recent investigations of the transport systems of micro organisms have implicated proteins to be intimately involved with a proline transport system in E. coli (Kaback and Stadtman, '66), a sulfate transport system (Pardee, '66) and a β-galactoside permease in E. coli (Fox et al., '67).

As a result of these and other observations the components of the membrane take on a central role in transport phenomena. Attempts have been made to isolate and associate membrane fractions with the reactive sites involved with sugar transport. One of the approaches was to isolate a lipid or lipid-soluble complex which would preferentially bind

glucose. LeFevre et al. ('64) demonstrated that phospholipids extracted from ghosts of human erythrocytes are capable of carrying glucose (labeled with carbon-14) from the dry state into the highly non-polar solvent hexane. Though this system lacks the specificity seen in normal sugar transport systems, it is interesting that this transfer of glucose can be inhibited in a manner similar to the inhibition of normal sugar transport. Although the phosphorus content of the extract is unchanged, lipids extracted from red cells which have previously been exposed to 1-fluoro-2, 4-dinitrobenzene (an irreversible and potent inhibitor of glucose transport capacity in the red cell) show a marked decrease in their ability to form the hexane-soluble complex with glucose.

The capacity of phospholipids derived from beef red cells (cells with extremely low glucose permeability) to bind hexoses was compared with that of the phospholipids derived from human red cells (Hobson and Laris, '66). The results indicated that: 1. Human and beef stromal phospholipids display equal abilities to carry glucose and mannitol into highly non-polar solvents, and 2. Glucose to which both cells are permeable is more effectively carried into hexane than mannitol, a sugar which penetrates neither cell. However, it is impossible to account for the vastly different hexose permeabilities of beef and human erythrocytes merely in terms of simple differences in the ability of the membrane phospholipids of these two cells to bind hexose.

Another approach towards isolating a carrier-type molecule was taken (Mawdsley and Widdas, '67) in the following manner: Human red cell ghosts were exposed to glucose labeled with carbon-14 and after a suitable period were extracted with lipid solvents. The extracts were fractionated on silicic acid columns. A peak identified as a triphosphoinositide (TPI) was associated with the radioactive peak and was thus presumably bound,

either physically or chemically, to glucose. Such a complex could be formed *in vitro* with pyridine as the solvent. Such *in vitro* labeling could be eliminated by pretreating the ghosts with DNFB or mercuric chloride. The authors suggest that it is this lipid fraction (TP1) which is a factor in the solubilization (LeFevre et al., '64 and Hobson and Laris, '66) of sugars in non-polar solvents.

Bobinski and Stein ('66) and Bonsall and Hunt ('66) in the attempt to isolate a glucose-binding component from human red cell membranes, prepared columns of membranes or membrane fractions immobilized on an absorbent such as celite or DEAE-cellulose. Radioactive sugar pairs were passed through such columns and by measurement of the effluent activity, it was possible to determine if glucose was being held back on the column in preference to a second sugar such as sorbose. Membranes prepared from DNFB-treated red cells were also used in these columns. The authors were able to demonstrate the appearance of a component associated with most of the glucose binding capacity of the erythrocyte membrane and suggest that this material may be responsible for glucose transport in the intact erythrocyte. However, like all methods which rely on cell destruction and subsequent isolation of a fraction, it is extremely difficult to show a correspondence between the physiologifal activity of an homogenate fraction and this same activity in the intact cell.

The other major approach toward eliciting information on non-electrolyte transport has been the study of the kinetics of non-electrolyte movement across the membrane of the intact cell and the effects of a variety of substances on such movement as was observed. These measurements were usually made on either net or exchange fluxes, but not on both fluxes using identical cell populations. The approach

taken in the work reported here has been to utilize intact cells and measure and analyze the effects of a number of different agents on both the exchange and maximal net fluxes obtained from identical cell populations. It was considered that the two fluxes presented an opportunity to analyze the carrier system under two different physical conditions - glucose-loaded and unloaded. Therefore the type of effect obtained with the different agents on the carrier states under loaded and unloaded conditions would yield a better insight to understanding the biochemical nature of the "carrier".

Insulin

As part of this approach the effect of insulin on glucose transport has been investigated. Levine et al. ('49,'50) demonstrated the ability of insulin to accelerate the permeability of cells to hexoses. Insulin has been shown to stimulate the transport of amino acids (Akedo and Christensen, '62) and their incorporation (Elsas et al., '67) into proteins in skeletal muscle. Insulin has been shown to affect the sodium flux in the toad oocyte (Bittar et al., '68), to possibly act on nucleotide transphorylases (Klachko, '66) by regulating the relative quantities of nucleotide triphosphates in the cell, to facilitate the transport of glucose, galactose and other sugars across the membranes of cardiac muscle (Morgan, et al., '65) and adipose cells (Rodbell, '67 and Blecher, '67). In the presence of insulin, glucose incorporation into glycogen is markedly increased (Søvik, '66); there is an increase in both membrane potential and membrane resistance observed with frog skeletal muscle in a glucose-free medium (deMello, '67), and there are indications of an involvement in the binding of transfer and messenger RNA to the ribosome (Stirewalt et al., '67).

The above is but a small sample of the vast number of effects in which

insulin has been implicated. In all of these investigations a mechanism concerning the primary site of action has been sought and the general consensus is more or less summed up by Levine ('65,'66) who believes that the primary site of insulin action is at the cell membrane. The manner in which insulin interacts with the cell membrane is as yet uncertain. However, a number of interesting observations have been made in this regard. Insulin has been shown to bind (Edelman et al., '63) to rat skeletal muscle cell membranes by means of electrovalent and covalent (disulphide) linkages. The evidence indicated an involvement of cyclic disulphide linkage of the insulin A chain and the sulphydryl groups on the receptor protein. Similar conclusions of a thiol-disulphide exchange reaction have come from others studying the binding of insulin to rat epididymal fat pads and hemidiaphragms (Fong et al., '62). The similarity of action (namely an increase in permeability) by phospholipase C, phospholipase A and insulin on the cell membranes of free adipose cells with respect to glucose entry has been demonstrated (Rodbell, '64; Blecher, '65 and Blecher, '66). It is suggested that these results offer evidence that plasma membrane phospholipids play a role in the facilitated transport of glucose. It is suggested (Blecher, '66) that limited hydrolysis of membrane phospholipids might transform membrane lipoproteins from laminar to globular configurations with a resultant increased permeability to small molecules. By comparison, phospholipase treatment of the red cell caused a marked decrease in the exchange diffusion of glucose (Odesser and Mawe, '67). No mention is made, (Blecher, '65) however, as to whether insulin might be involved in a similar manner. There is a possibility that an alteration of the configuration of plasma membrane lipoproteins could occur by formation of complexes between phospholipases, calcium and their phospholipid substrates (Blecher, '65). A similar interaction between insulin, zinc and membrane lipoprotein has been proposed (Krahl, '61).

In a series of reports, the nature of the insulin receptor site in muscle has been investigated (Rieser, '65; Rieser, '66; Rieser, '67 and Rieser, '67a). In these reports, the author has demonstrated the proteolytic-like activity of insulin and also the insulin-like activity of several proteolytic enzymes. It is suggested that insulin may be compared to an incomplete or partial proteolytic enzyme, which, for its activity to be expressed, must in some manner combine or orient with a complement of amino acid residues (to be found on the membrane surface) and in this manner become an entire functional catalytic unit. This approach is an extension of the hypothesis advanced by Hofmann ('60) for peptide hormone formation where a cell receptor is pictured as an incomplete enzyme with the hormone supplying the missing part. Rieser ('67) has also presented preliminary evidence that tryptophan (an amino acid not found in insulin from any species) may be a part of the insulin receptor site.

Another interesting proposal for a mechanism of insulin and other disulphide hormone action is presented by Robinson ('66). The author suggests that there may be an interaction between the sulphydryl groups of the hormone and the cis double bonds in lipid fatty acids. He proposes that such an interaction could affect the packing of the hydrocarbon chains, such that there would be an alteration in the surface charge properties as well as in protein conformation. Such changes, if they occurred, could in turn affect membrane permeability.

The evidence for the facilitated transport of glucose as summarized by Stein ('64) has been mentioned previously. The effect of insulin on some of these parameters has been discussed by Henderson ('64). For example, Park et al., ('65) demonstrated the existence of a definite degree of stereospecificity in the glucose transport system in perfused heart experiments and in addition the fact that insulin stimulates the sterospecific

system only.

The interaction of insulin and other protein hormones with the sugar transport system in human red cells is one of the aspects of the work reported here. The transport of glucose across the membrane of the normal human red cell has been considered to be unaffected by insulin (Pletscher et al., '55). However, when the red cells are exposed to chymotrypsin, it has been reported that glucose flux is enhanced by insulin (Rieser and Rieser, '64). The elevation of the maximal net flux of glucose in normal human erythrocytes by insulin alone has been reported (Zipper and Mawe, '67), and further comment about this work and other related items will be held for a later section.

MATERIALS AND METHODS

The materials used in these investigations and the sources from which they were obtained are as follows: Bovine insulin (25 units/mg), porcine insulin (23 units/mg), insulin A-chain oxidized, insulin B-chain oxidized, insulin S-sulfo A-chain, insulin S-sulfo B-chain, L-epinephrine, adrenocorticotropic hormone (ACTH) (porcine pituitary, 200 I.u./mg), were from Mann Research Laboratories, N.Y.C., N.Y.; Bovine insulin (24 units/mg), oxytocin, synthetic (10 units/mg), vasopressin, synthetic (150 units/mg), glutathione oxidized form, glutathione reduced form, phospholipase C, β-D(+) glucose, p-chlormercuriphenyl sulfonic acid (PCMBS), polymixin B sulfate, protamine sulfate (grade I) were from Sigma Chemical Co., St. Louis, Mo.; Phospholipase A (Naja naja venom), phospholipase B (bovine pancreas), phospholipase D (0.5 units/mg, cabbage), Cottonmouth venom, were from Pierce Biochemical Co., Rockford, Ill.; L-cysteine, L-cysteic acid, and L-cystine were from Calbiochem, Los Angeles, Calif.; Bovine insulin, sterile (10 units/ml.) was obtained from Squibb and Co., N.J.; porcine insulin-I^{131} was from Abbott Laboratories, Chicago, Ill.; Chlormerodrin (neohydrin) was a gift from Dr. H. L. Friedman of the Lakeside Laboratories, Milwaukee, Wisc.; Chlormerodrin labeled with Hg^{203}, PCMBS labeled with Hg^{203} and D-glucose-C^{14}-UL were supplied by ICN Corp., City of Industry, Calif. The γ-poly glutamic acids were isolated from B. subtilis and were a gift from D. J. O'Connell of the Veterans Administration Hospital, Brooklyn, N.Y. All other materials were of reagent grade. Water was triply distilled, once from a tinlined container, and twice from glass. The materials listed above were stored in the presence of desiccants at the temperature (4°C or -20°C) which was considered to be the best for maximum stability. Unless otherwise noted, solutions of these materials were prepared just prior to their use and were then discarded.

Samples containing carbon-14 were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 4322. The background averaged about 20 cpm. The scintillation mixture consisted of p-dioxane; ethoxyethanol:xylene in the proportion of 3:3:1. This mixture (Bruno and Christian, '61) was prepared to contain 8% naphthalene, 1% 2,5-diphenyloxazole (PPO), and 0.05% 1, 4-bis-2-(4-methyl-p-phenyloxazolyl)-benzene (Dimethyl POPOP). PPO and Dimethyl POPOP were obtained from Packard Instruments Co., Downers Grove, Ill. Ethoxyethanol, p-dioxane (spectroquality grade), xylene, naphthalene (recrystalized from alcohol) were from Matheson, Coleman and Bell, Inc., Rutherford, N.J. This scintillation mixture is capable of accepting up to 20% of its volume of an aqueous sample, will not freeze at the normal operating temperatures utilized in liquid scintillation counting and has a good counting efficiency by comparison with other mixtures. Samples labeled with Hg-203 or I-131 were counted in a Packard model 2001 Spectrometer well counter system. The background level with this instrument ranged 60-80 cpm.

Measurements of pH were made on a Radiometer pH meter, model TTT-C-1 with a Corning combination electrode, type 476020. Osmolarities were determined by the freezing point depression method utilizing an Advanced Instrument Osmometer, model 31-LAS. Hematocrits were determined with the use of a Spinco micro-centrifuge (Microfuge) Beckman Instr. Model 15-2A. Tubes, 1 mm in diameter, were partially filled with a sample of the blood suspension. One end of the tube was sealed with clay and the tube centrifuged for 2 minutes at top speed in the micro-centrifuge. The ratio of cells to the total sample volume was determined with the aid of an IEC micro-capillary reader.

Flux Measurements

The experimental arrangement for rapidly separating cells from super-

natant fluid was as follows: A stainless steel Swinny filter holder (Millipore #XX30 01200) is fitted at its distal end to a 2 ml. syringe, and at its proximal end to a 14 gauge cannula. The teflon gaskets and wire (stainless) screen in the filter holder are arranged in a manner to support a millipore filter when suction is applied upon drawing up on the syringe. The filter (Millipore #SMWP 013) is a 13 mm diameter cellulose filter with a mean pore size of 5.0 μ ± 1.2 μ. The red cells are efficiently held back by the filter. Thus, when a sample from a dilute red cell suspension is rapidly withdrawn, the cells are held on the filter, while a cell-free supernatant rapidly collects in the syringe. By quickly disengaging (less than 0.5 sec.) the syringe from the filter holder, the cell-free supernatant is physically removed from any contact with the filter containing the cells and the possibility of secondary back fluxes occurring is completely eliminated.

Red blood cells were obtained, unless otherwise indicated, from healthy human donors. Blood (15-20 ml.) was withdrawn by venipuncture and collected in a tube containing sodium citrate (5 mg/ml. blood). The citrated blood was poured through three layers of gauze, centrifuged at 800 xg for 10 minutes and the plasma and white cells removed. The cells were washed three times at 4°C with a 0.075 M glucose-saline solution, adjusted to pH 7.3 with phosphate buffer. The cells were collected each time by centrifugation at 800 xg for 10 minutes. When not used shortly after the last wash (experiments denoted as day 1), the cells were stored in the glucose-saline-phosphate solution at 4°C. Prior to being used after storage (experiments denoted as day 2 - 24 hours storage ... etc.), the cells received one additional wash with the glucose-saline-phosphate solution. The primary buffer solutions utilized in most of the experiments were: 1. a saline 0.01M phosphate solution adjusted to a pH of 7.3 and which was 295-310 milliosmoles per liter and 2. a saline 0.01M phosphate solution

adjusted to pH 7.3, containing 0.075M glucose and which was 390-400 milliosmoles per liter.

After the final wash, aliquots of packed cells were equilibrated with glucose-C^{14}-UL. An aliquot of 25 μl of glucose-C^{14}-UL (125 μcuries/ml.) was added to 0.5 ml. of a thick cell suspension (hematocrits 75-85%) and the suspension was allowed to equilibrate for 30 minutes at 22-24°C. The equilibrated cells are now in a position to be treated with any of the compounds which have been listed above. The concentrations which were used for each compound will be given later when the results obtained with each agent are presented. On the whole, the attempt was made to keep all additive volumes to a minimum level of between 25 and 50 μl. The treated cells were incubated for specified times and at specified temperatures (given later in the text when the individual compounds are considered). Concomitantly, non-treated 0.5 ml. aliquots of thick red cell suspensions (hematocrits 75-85%) are carried through the entire procedure. Only a similar quantity of the solution used to dissolve the particular agent added to the treated cells is added to these cells. In this way, flux values are obtained for treated and non-treated cells derived from the same cell population.

After the prescribed period of incubation has elapsed, 10 μl. of the red cell suspension is removed and placed on a small glass ladle. With the use of the glass ladle, the blood sample is rapidly (with stirring) introduced into a beaker containing 10 ml. of a buffer solution. When net flux measurements are made this buffer solution is saline-phosphate pH 7.3, 295-310 milliosmoles per liter. Under these conditions, the glucose concentration in the cell's environment is reduced to 0.015×10^{-3}M, a value which does not exceed or even approach any of the suggested Km values (Sen and Widdas, '62) for the carrier site. The conditions for the measure-

ment of the maximal net flux are therefore met (Rosenberg and Wilbrandt, '55). When exchange flux measurements are to be made this buffer solution is 0.075 M glucose-saline-phosphate pH 7.3, 390-400 milliosmoles per liter. An electric timer is actuated as the red cells are introduced into the solution in the beaker. Samples (approximately 0.5 ml.) are rapidly withdrawn (at 4-5 second intervals) with the aid of the Swinny filter-syringe device. In this way five cell-free samples are rapidly obtained. The first sample is usually obtained within 1.5-3 seconds after the introduction of the cells into the buffer solution. Subsequent samples are usually obtained at 5-second intervals. The sampling time is consistently considered as that time after the sample has been withdrawn, when the syringe is separated from the filter holder. After the rapid sampling period is over the beaker containing the diluted red cell suspension is set aside for 30-60 minutes. By this time glucose-C^{14} is uniformly distributed between cell water and medium and a final cell-free sample (the infinity sample) is taken. Aliquots of 0.1 or 0.2 ml. of the cell-free samples are placed in vials containing 10 ml. of the scintillation mixture previously described and the samples are counted for the two 10-minute cycles in a Packard liquid scintillation spectrometer.

The radioactivity counted in the supernatant samples comes from the glucose-C^{14} which leaves the cells plus the initial radioactivity which is carried over in the extracellular phase of the packed red cells. This latter additional radioactivity is constant in all six samples. The sixth sample, the infinity or equilibrium sample, is used to determine the maximum amount of glucose-C^{14} which will leave the red blood cells.

In order to make a valid comparison between exchange and maximal net fluxes of both treated and non-treated cells, all measurements were made on cells from the same blood sample within 15-30 minutes of one another.

At time 0, with the immersion of the ladle containing the red cell suspension, the specific activity of the cells' environment is reduced to less than .01/10.01 or at least to 9.99×10^{-4} of its value before immersion of the ladle. The specific activity of the cells is unchanged. Under exchange flux conditions, the glucose concentration inside and outside the cells remains constant through the experiment, while under net conditions, within 0.5 seconds after the ladle's immersion, the glucose concentration within the red cells is reduced due to the entry of water. This reduction depends upon the difference between the osmolarities of the incubation solution and of the external medium. If the cells were previously equilibrated with 0.075 M glucose-saline-phosphate, the concentration of glucose would be reduced to 310/390 or 0.8 of that in the equilibrating solution. Therefore, at time 0, there would be a concentration gradient between the cell and the medium.

Theoretical Considerations

The conditions under which the equilibrium flux were measured were the same as that of a two-compartment closed system previously discussed by Solomon ('49) and Sheppard ('62). Under these conditions the cell water forms one compartment while the medium water makes up the second compartment. Initially, almost the total amount of the tracer is present in the cell water compartment. Under these conditions labeled material is moving one way out of the cell into the medium water compartment while unlabeled material is moving out from the medium water compartment into the cell water compartment. The level of the labeled material follows an exponential decline such that the rate of loss of the label is always proportional to the amount present. As a result, a plot on semi-log paper of the loss of label with time will result in a straight line, the

half-time (t 1/2) being that time which it takes for the reduction of the quantity of the label to 50% of its initial level.

The kinetic analysis which follows has been taken from Mawe and Hempling ('65). The kinetics of the exchange is described by equation 1.

$$\ln\left[1 - \frac{\text{Spec. Act. medium, } t}{\text{Spec. Act. medium, } \infty}\right] = -P\left(\frac{1}{S_1} + \frac{1}{S_2}\right)t$$

where P = flux of glucose in moles per liter cell water-sec.

S_1 = cell compartment size of glucose in moles/liter

S_2 = medium compartment size of glucose in moles/liter

The specific activity may be defined as the cpm/mole glucose. Since the moles of glucose in each compartment would be the same at time t as at time ∞, equation 1. can be rewritten as:

$$(2) \quad \ln\left[1 - \frac{\text{cmp/ml. medium, } t}{\text{cpm/ml. medium, } \infty}\right] = -P\left(\frac{1}{S_1} + \frac{1}{S_2}\right)t$$

Since the compartment size of glucose in the medium S_2 is much larger than the cell compartment S_1, $1/S_2$ is very small and may be neglected in the calculation of the flux. As such equation 2. reduces to:

$$(3) \quad \ln\left[1 - \frac{\text{cpm/ml. medium, } t}{\text{cpm/ml. medium, } \infty}\right] = -(P/S_1)\ t$$

A plot of the values from the left hand term of equation 3. against time (t) will yield a straight line, whose slope m is P/S_1. Therefore the flux, P, will equal the slope m multiplied by S_1: or P can be determined as shown in equation 4.

$$(4) \quad P = \frac{0.693}{t\ 1/2} \times S_1$$

Widdas ('54), LeFevre and McGinniss ('60) and Wilbrandt ('61) agree that the following equations describe the kinetics of net glucose exit:

$$(5) \quad -\frac{d(G)c}{dt} = \frac{d(G)m}{dt} = Vmax \left[\frac{[Gc]}{Km + [Gc]} - \frac{[Gm]}{Km + [Gm]} \right]$$

where Vmax may be defined as the maximum rate of movement of which the carrier system is capable.

(G)c = the amount of glucose in the cell compartment

(G)m = the amount of glucose in the medium compartment

[Gc] = the concentration of glucose in the cell water

[Gm] = the concentration of glucose in the medium water

Km = the dissociation constant of the glucose-carrier complex

when [Gm] << Km, [Gc]*, equation 5. simplifies to:

$$(6) \quad \frac{d\,(G)m}{dt} = \frac{Vmax\,[Gc]}{Km + [Gc]}$$

If [Gc] >> Km* then equation 6. becomes:

$$(7) \quad \frac{d\,(G)m}{dt} = Vmax = -\frac{d\,(G)c}{dt}$$

where equation (7) is a description of maximal net flux. We can write:

$$(8) \quad Vmax = -\frac{(dR/dt)\ cell}{Ac} = \frac{(dR/dt)\ medium}{Ac}$$

where (dR/dt) medium = cpm glucose entering the medium from the cells and is obtained from the slope of curves similar to those shown in Figure 6.

Ac = [cpm glucose in the cell/amount of glucose in the cell]

Since all the radioactivity found in the medium at infinity, $cpm_{t=\infty}$, came from the cell with the exception of that amount which was present as an

*As noted in the Material and Methods section, the initial concentration of glucose in the cell is 0.075 M and the final concentration of glucose in the environment is 0.015 x 10^{-3} M. The Km for glucose transfer has been reported as 1-5 x 10^{-3} M. Therefore, the restrictions [Gm] << Km, [Gc] and [Gc] >> Km are met and the equation 10. may be applied to the experimental data.

extracellular contaminant when the packed cells were mixed with the environment, we can write*:

$$(9) \quad (\text{cpm glucose in the cell})_{t\,=\,0} = (\text{cpm in the medium})_{t\,=\,\infty} - (\text{cpm in the medium})_{t\,=\,0}$$

The amount of glucose present when t = 0, when normalized to an isosmotic cell volume, is a concentration and is equal to 0.075 M in these experiments. Therefore:

$$(10) \quad V_{max} = \frac{(\text{slope of the curve, cpm/sec})\,[0.075\text{ M}]}{[(\text{cpm})_{med.\ t\,=\,\infty} - (\text{cpm})_{med.\ t\,=\,0}]}$$

Binding Experiments

In another series of experiments, the degree to which certain agents remain bound to the red cell under a variety of conditions and after extensive washings was investigated. In these experiments, the red cells were obtained, washed and equilibrated with cold glucose exactly as previously described. Glucose labeled with carbon-14 was not used. The washed cells were incubated with the agent under investigation for a given time and at a given temperature (the data are presented with the individual experiments reported later). The agents studied were PCMBS labeled with Hg-203, chlormerodrin labeled with Hg-203 and porcine insulin labeled with I-131. The treated cells were washed with either saline-phosphate at pH 7.3 or 0.075 M glucose-saline-phosphate at pH 7.3 Aliquots of the wash supernatants (1 ml.) as well as the washed packed red cells were counted in a well counter. The entire quantity of packed cells was counted after the final wash, no transfer or dilution being required. Counting

*to be strictly true it would be necessary for $[Gc]_{t\,=\,\infty} = 0$. However, since the volume of the environment is so much greater than the volume of cells which are added, the $[Gc]_{t\,=\,\infty}$ is sufficiently close to zero as to make equation 9. virtually correct.

standards containing known quantities of the radioactive agent being used were prepared at the same time as additions were made to the red cells. Those standards were counted along with the experimental samples and inasmuch as the results were expressed in terms of percentages based on known quantities which were added initially to the red cells, it was not necessary to consider physical decay in calculating the results from the data so obtained.

Hemolysis Experiments

A few experiments were devoted to the measurement of hemolysis obtained with phospholipase C in the presence of insulin. In these experiments isotopes were not utilized. Red cells were obtained, washed and equilibrated with glucose as previously described. Aliquots of 0.5 ml. of packed red cells (hematocrits 70-80%) were exposed for 20 minutes to 25 μl. volumes of phospholipase C (Phl-C) solutions whose concentration ranged from 5×10^{-3} to 0.1 mg Phl-C per 25 μl. Red cells were either first treated with 25 μl. of insulin (0.06 μMoles) for 30 minutes prior to their exposure to Phl-C or were exposed only to Phl-C. The cells were then centrifuged at 25000 xg for 15 minutes, a 25 μl. sample of the supernatant was removed, diluted to 10 ml. with water and the absorption determined at 540 mμ in a Klett colorimeter. The values so obtained were compared to a value representing 100% lysis of an identical 0.5-ml. packed red cell aliquot.

Flux Measurements-Special Conditions

Inasmuch as there is a difference of tonicity between the solutions used to measure net and exchange fluxes, a number of experiments were arranged in a manner such that this difference in tonicity was eliminated. In these instances sodium chloride was added to the normal saline-phosphate solution pH 7.3, 295-310 milliosmoles per liter to bring the con-

centration to 370-380 milliosmoles per liter, the pH remaining at 7.3. Thus, measurement of the net flux occurred at an almost identical tonicity as that of the exchange flux, the sole variable being the presence of equal concentrations of glucose on both sides of the cell membrane in one case (exchange) and the lack of glucose on one side in the other case (net).

Since all of the data obtained on flux measurements will be presented in tabular summary, it would be repetitious to include a graphic representation for each series of experiments. Nevertheless, it may be of some interest and possible value to see an example of the graphic display. Therefore, in Figure 6 there are plotted three curves showing the measurement of the maximal net flux in a) non-treated normal human red cells, b) insulin-treated normal human red cells and c) PCMBS-treated normal human red cells. In each case the counts/min (cpm) obtained at sampling time t is plotted against the sampling time t. The value for the cpm/sec is obtained from the slope and the cpm, medium, t = 0 is obtained from the intercept of the curve with the ordinate. Figure 7 shows the measurement of the exchange flux in a) non-treated normal human red cells, b) insulin-treated normal human red cells and 3) PCMBS-treated normal human red cells. The values of one minus the ratio of the cpm at sampling time t to the cpm of the sample at infinity (30-60 min) are plotted against the sampling time t. The half time (t 1/2), i.e., the time which it takes for the reduction of the quantity of the label to 50% of its initial value, is determined from the curve and this value is in turn utilized to determine the flux. In these two figures, examples of control values, activated fluxes, and inhibited fluxes are presented.

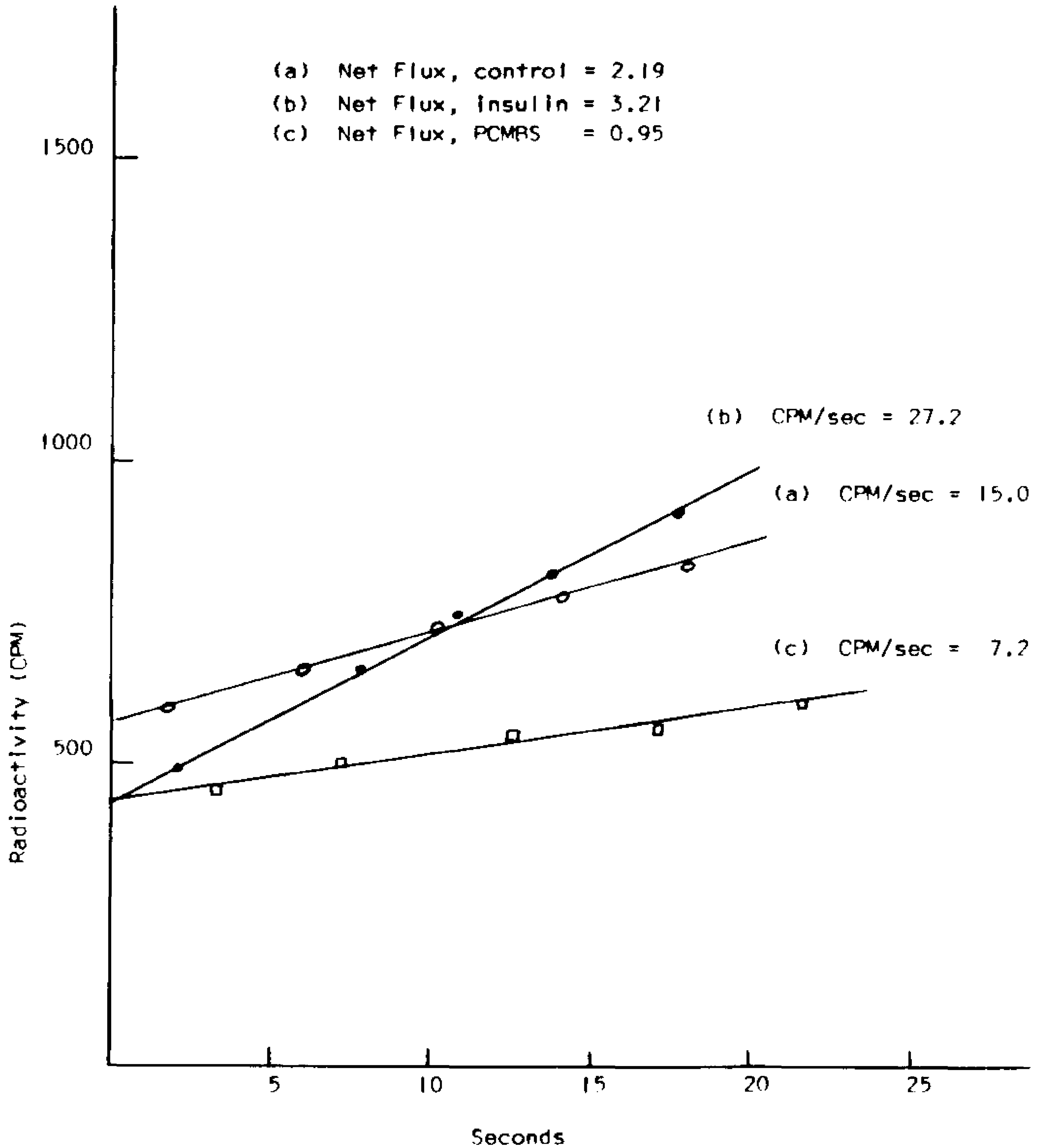


FIGURE 6. A comparison of the maximal net flux of glucose-C^{14}-UL in non-treated, insulin-treated and PCMBS-treated cells. Fluxes are expressed as millimoles per liter cell water-sec.

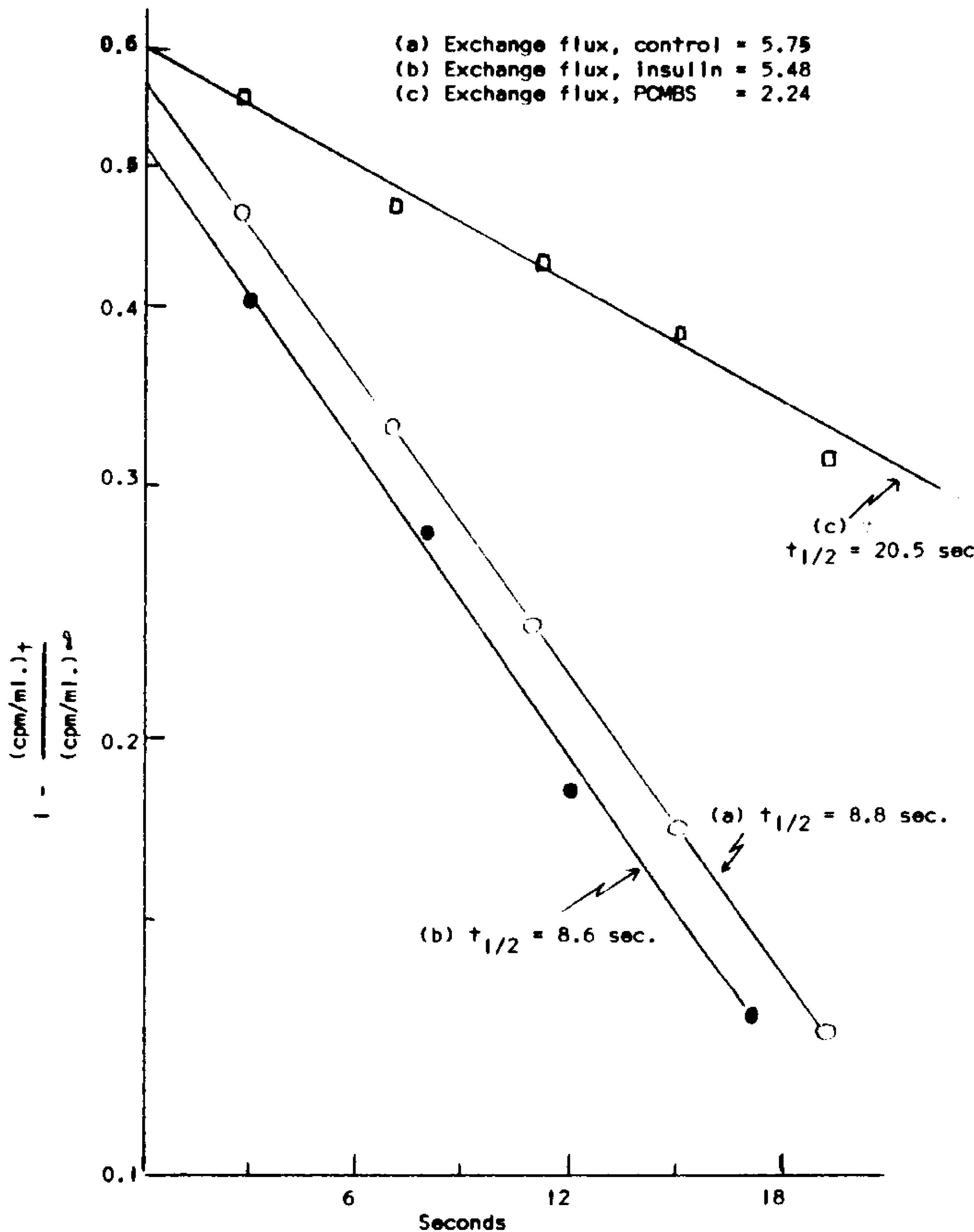


FIGURE 7. A comparison of the exchange flux of glucose-C^{14}-UL in non-treated, insulin-treated and PCMBS-treated cells. Fluxes are expressed as millimoles per liter cell water-sec.

As part of the calculations for determining fluxes under maximal net and exchange conditions, changes in the cell volume during the incubation period, due to the addition of small volumes containing the agents which were tested, must be taken into account. To determine volume dilution corrections, the following series of relationships are used:

a) ml. cells = vol. of suspension (0.5 ml.) x hematocrit (80%) = 0.400

b) ml. plasma H_2O = vol. of suspension (0.5 ml.) - ml. cells 0.4 ml. = 0.100

c) cell H_2O = 0.70 x ml. cells (0.4 ml.) = 0.280

d) total H_2O in initial vol. of suspension = plasma H_2O (0.100) plus cell H_2O (0.280 = 0.380

e) total H_2O in the system = plasma H_2O plus cell H_2O (0.380) plus ml. additional H_2O added (0.050) = 0.430

f) vol. dil. corr. = $\frac{\text{total } H_2O \text{ in initial vol. of cells}}{\text{total } H_2O \text{ in system}} = \frac{0.380}{0.430}$ = 0.884

The value for the vol. dil. corr. is used in the following equations for determining the flux value.

$$\text{Exchange flux} = \frac{0.693 \times \text{molarity glucose soln.} \times \text{vol. dil. corr.}}{t\ 1/2}$$

$$\text{Net flux} = \frac{(\text{cpm/sec})\ [0.075\ M]\ (\text{vol. dil. corr.})}{[(\text{cpm})_{\text{med. } t=\infty} - (\text{cpm})_{\text{med. } t=0}]}$$

Statistical Analysis

The data which were obtained in the various experiments were where possible treated in the following manner. Data obtained under a given set of similar conditions were grouped and averaged. The standard deviation and the standard error of the mean were determined. Values exceeding the limit of $\pm$ 1.96 S.D. were discarded. The means from different groupings were compared for the presence or lack of significant difference by means of the student's t test. In this test if $p > .05$ was found for a

given comparison, it was considered that within each group there existed values which could be placed with equal probability in one group or the other and that there was therefore no significant difference between the two values in question. The following equations are utilized in the statistical analysis employed in this paper: The variance or mean square deviation is determined by equation 10.

(10) Variance = $(X - \bar{X})^2 / N-1$ and this value is used to determine the standard deviation (S.D.) in equation 11.

(11) $S.D. = \sqrt{Variance} = \sqrt{(X - \bar{X})^2 / N-1}$

The standard error of the mean is determined by equation 12.

(12) $S.E. = S.D./\sqrt{N} = \sqrt{\dfrac{(X - \bar{X})^2 / N-1}{\sqrt{N}}}$

In order to compare the significance between two means with the aid of the t test, the standard error of difference is determined according to equation 13.

(13) $S.E.\ dif. = \sqrt{S.E._1^2 + S.E._2^2}$

and is in turn utilized in equation 14 to determine t

(14) $t = \bar{X}_1 - \bar{X}_2 / S.E.\ diff.$

The probability (p) of significance is determined from a standard table by means of the value for t and the value for n where $n = [(N_1 - 1) + (N_2 - 1)]$.

Structural Formulas

The structural formula for insulin as given by Sanger et al ('55) is shown in Figure 8. When insulin is subjected to sulfitolysis (Dixon and Wardlaw, '60), the α and β chains are split and the resulting products are shown in Figure 9a and 9c. Under these conditions the interchain disulfide groups are split and S-Sulfo groups are formed. The intra disulfide linkage on the alpha chain remains intact. This disulfide linkage will also be split if sulfitolysis takes place in the presence of 8 M urea (Fig. 9b). The S-Sulfo chains utilized in the work reported here are the ones prepared without urea and are the ones shown in Figure 9a and 9c. The alpha and beta chains of insulin obtained by oxidative methods (Ryle and Sanger, '55) are shown in Figure 10. As can be seen, all the disulfide linkages have been broken and replaced by sulfonic acid groups. The structural formulas of beef oxytocin and beef vasopressin are shown in Figure 11. These two cyclic hormones are both of low molecular weight and contain a disulfide group as an inherent part of their structure. The molecules, glutathione reduced form, glutathione oxidized form, cysteic acid and L-cysteine are shown in Figure 12. In Figure 13 the structural formulas of ACTH and poly-γ- glutamyl acid are presented. The ACTH used in the experiments reported here was of porcine origin, while the structural formula shown in Figure 13 is that of human ACTH. Both molecules are identical except for the portion of the chain which is in brackets. The two mercurials, chlormerodrin and PCMBS are shown in Figure 14.

Phosphatidyl serine was chosen to represent a typical phospholipid in order to demonstrate where the various phospholipases attack such a molecule. The molecule, the points of attack, the phospholipases and the end products of hydrolysis are shown in Figure 15.

NH_2 — S — S — NH_2 NH_2 NH_2

Gly-Ile-Val-Glu-Glu-Cy-Cy-Ala-Ser-Val-Cy-Ser-Leu-Tyr-Glu-Leu-Glu-Asp-Tyr-Cy-Asp

S S

S S

NH_2 NH_2

Phe-Val-Asp-Glu-His-Leu-Cy-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cy-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr

Ala-Lys-Pro

FIGURE 8. The structural formula of bovine insulin (M.W. 5800) according to Sanger et al., ('55)

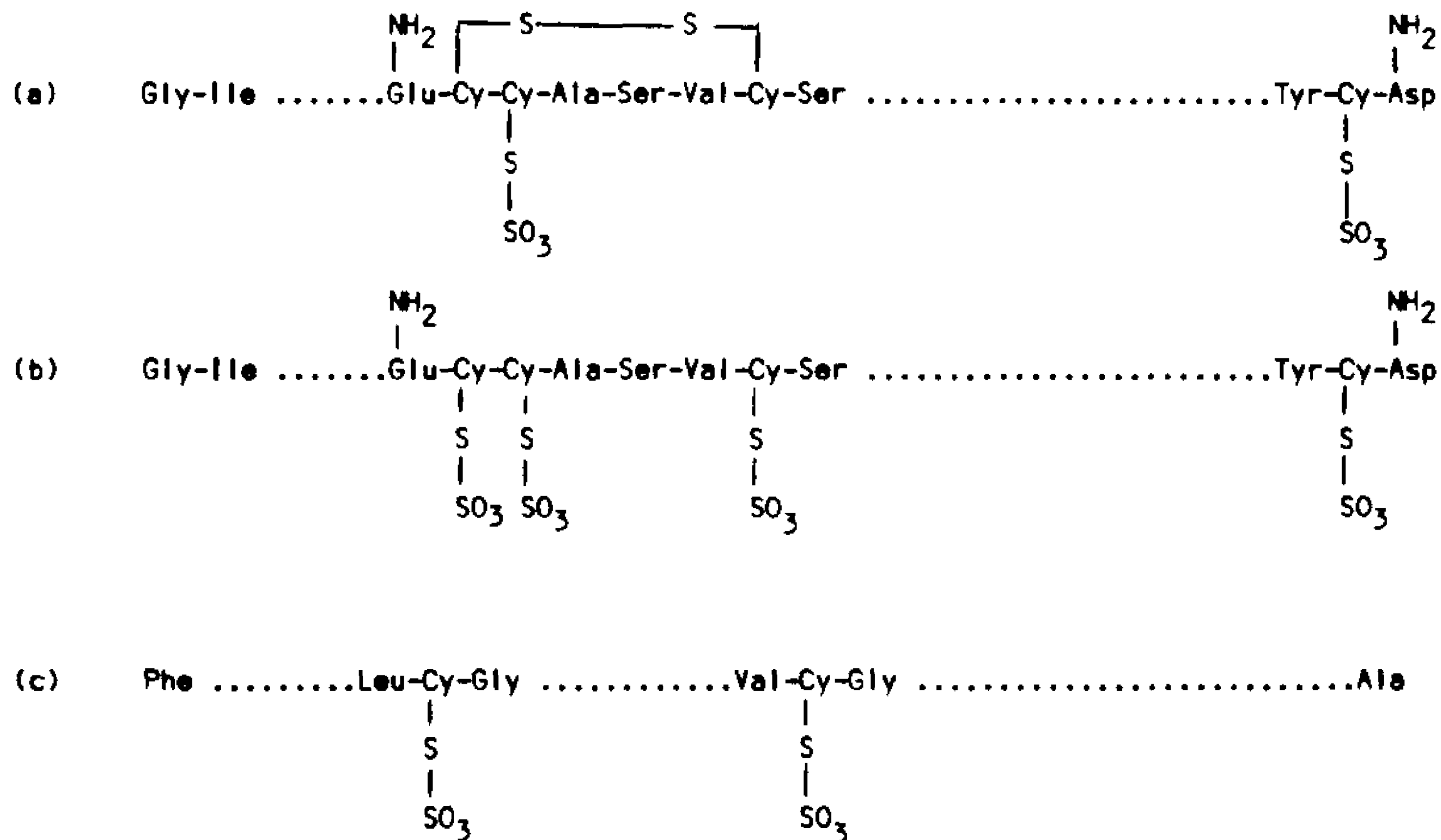


FIGURE 9. Insulin chains prepared by sulphitolysis. (a) S-Sulfo-A-Chain M.W. 2500; (b) S-Sulfo-A-Chain M.W. 2670 (prepared in the presence of 8M urea); (c) S-Sulfo-B-Chain M.W. 3490.

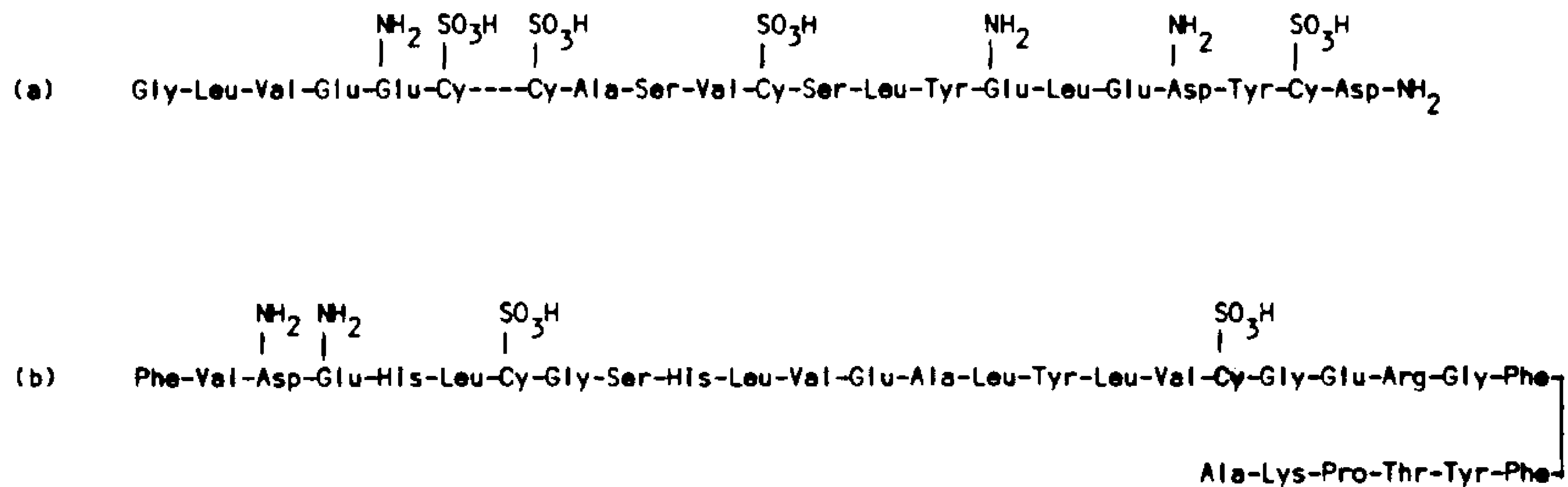
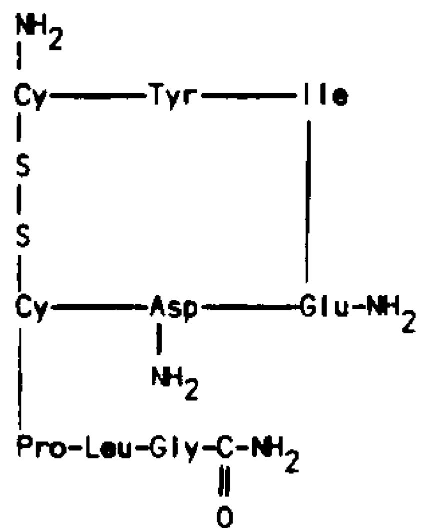


FIGURE 10. (a) Oxidized bovine insulin A-chain M.W. 2530; (b) Oxidized bovine insulin B-chain M.W. 3420.



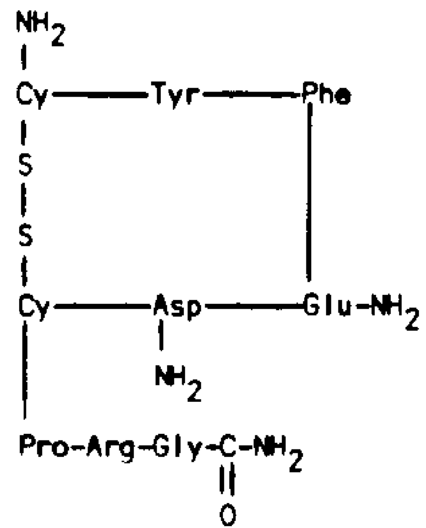
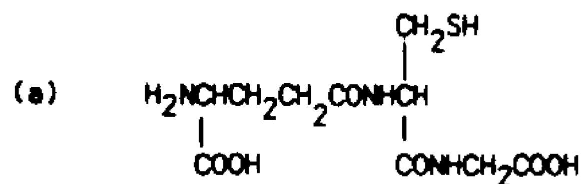
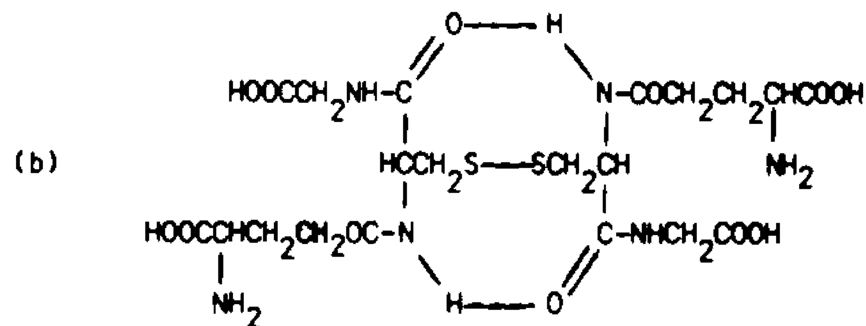


FIGURE 11. (a) Beef oxytocin M.W. 1080; (b) Beef vasopressin M.W. 1150.

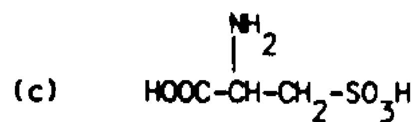
(a)



(b)



(c)



(d)

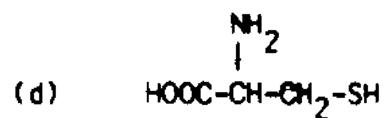
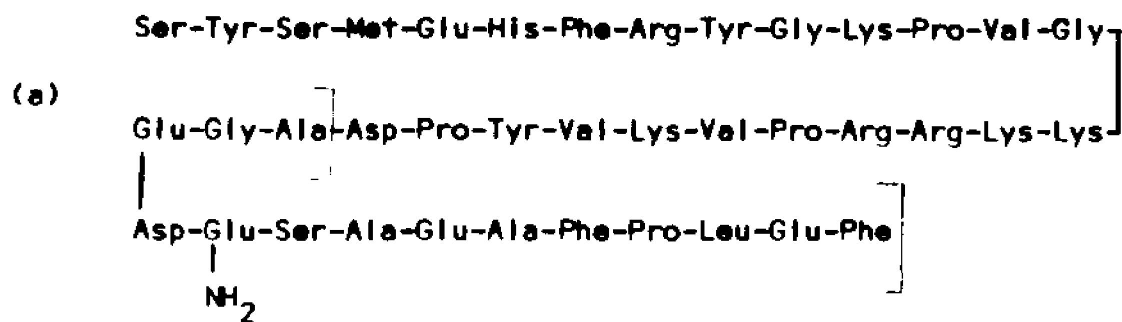


FIGURE 12. (a) GSH M.W. 307; (b) GSSG M.W. 612; (c) Cysteic acid M.W. 169; (d) Cysteine M.W. 121.

(a)



(b)

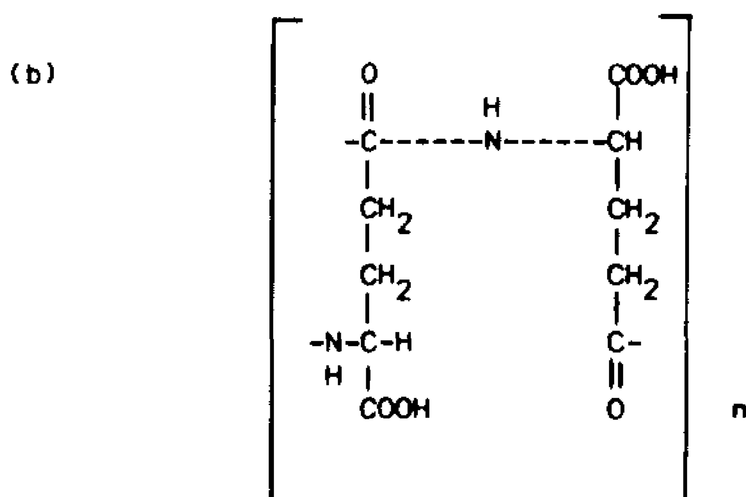
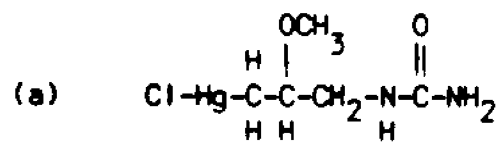


FIGURE 13. (a) Adrenocorticotrophin (Porcine) (ACTH) M.W. 3500
(b) poly- -glutamyl acid n=9, 30, 45. M.W. 2300; 8100; 11600.



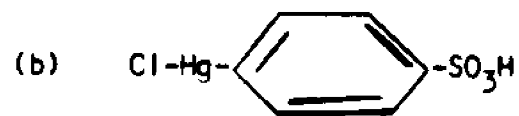
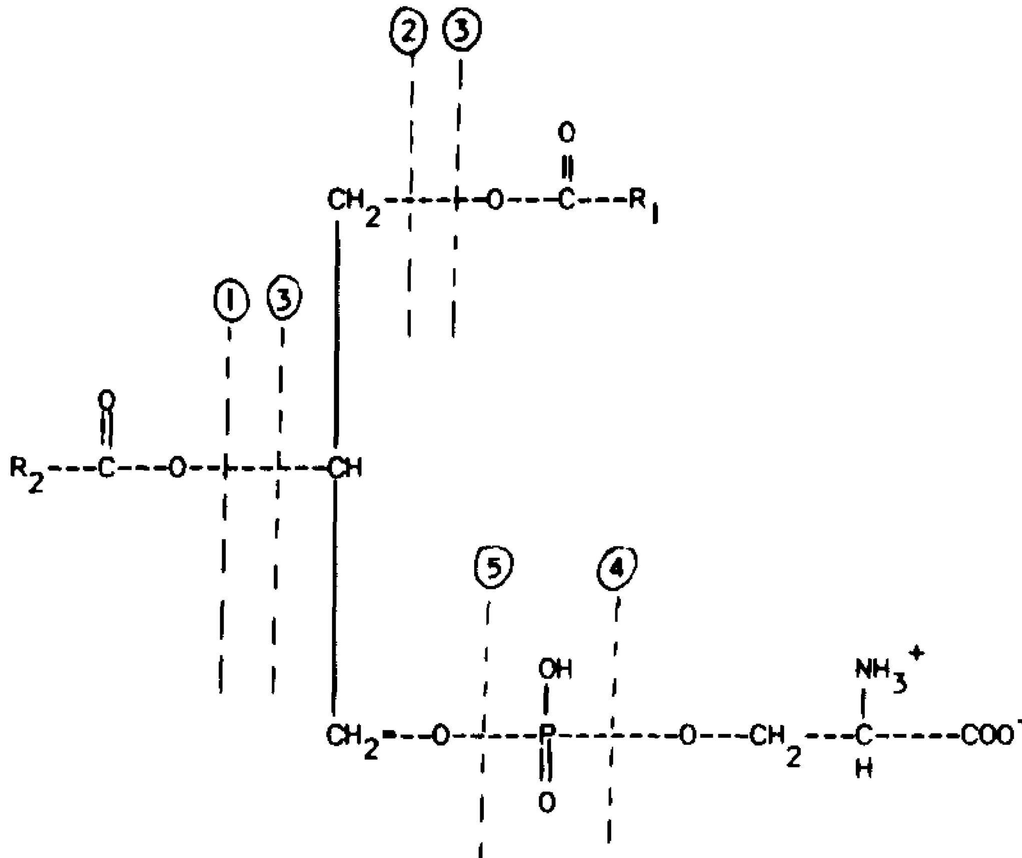


FIGURE 14. (a) Chlormerodrin M.W. 367; (b) PCMBS M.W. 393.



Enzyme	End Products
1. Phospholipase A	R_2COOH Lysophosphatide
2. Lysophophatidase	R_1COOH Glycerol Phosphoryl Base
3. Phospholipase B	R_1COOH R_2COOH Glycerol Phosphoryl Base
4. Phospholipase C	Phosphatidic Acid Base
5. Phospholipase D	α,β-diglyceride Phosphorylated Base

FIGURE 15. The structural formula of phosphatidyl serine and the points at which different phospholipases act.

RESULTS

Controls

Since flux measurements were made on non-treated cells in every experiment reported here, it was therefore possible to accumulate a large quantity of such data on cells from a group of normal donors. The net and exchange fluxes of seven such normal donors are listed in Table I. With this data, it is possible to analyze the net and exchange fluxes with respect to variation between donors and to variation between cells stored for different time intervals (e.g., day I through day 3). Day I refers to data obtained from freshly-drawn cells, usually within 8 hours after bleeding. Day 2 represents data from cells 24 hours old and by day 3 the cells are 48 hours old. The third column in each section of Table I shows the value for the E/N ratio (exchange flux/net flux). This value has been shown (Mawe and Hempling, '65 and Levine et al., '65) to range between 2 and 2.5 for normal erythrocytes.

The data presented in Table I has been analyzed by means of the student's t test for significant differences between selected pairs. The result of this analysis between selected pairs is given in the appendix. The comparison of the overall average values for the net and exchange flux by the day shows that the net flux values decline by 10% over a 48-hour period, and the exchange flux value declined 13% in the first 24-hour period and a total of 16% over the 48-hour period during which the cells were usually stored.

Insulin, Insulin Derivatives, Peptides and Small Proteins

The individual flux values obtained under maximal net and exchange conditions from non-treated and insulin-treated cells are shown in Table 2.

TABLE 1. The average net and exchange fluxes of glucose-C^{14}-UL obtained with the red cells of normal male donors. Measurements were made on cells which were freshly drawn (day 1), had stood 24 hours (day 2) or 48 hours (day 3) in 0.075 M glucose-saline-phosphate pH 7.4 at 4°C. Flux values are expressed as millimoles per liter cell water-sec.

	Day 1			Day 2			Day 3		
Donor	Net Flux ± S.E.	Exchange Flux ± S.E.	E/N	Net Flux ± S.E.	Exchange-Flux ± S.E.	E/N	Net Flux ± S.E.	Exchange Flux ± S.E.	E/N
1	2.32 ± .04	6.05 ± .11	2.61	2.04 ± .03	5.45 ± .12	2.67	2.02 ± .06	5.28 ± .24	2.61
2	2.41 ± .04	6.97 ± .39	2.89	2.17 ± .05	5.47 ± .15	2.52	2.54 ± .08	5.93 ± .52	2.33
3	2.36 ± .11	6.46 ± .37	2.74	1.94 ± .15	5.38 ± .47	2.77	2.18 ± .12	5.05 ± 1.26	2.32
4	2.28 ± .07	5.78 ± .26	2.53	2.19 ± .07	6.66 ± .82	3.04	-	-	-
5	2.47 ± .09	6.64 ± .31	2.70	2.54 ± .07	6.10 ± .11	2.40	2.35 ± .30	4.36 ± .25	1.86
6	2.27 ± .07	6.99 ± .26	3.08	2.50 ± .17	6.50 ± .27	2.60	3.55 ± .10	6.30 ± .67	2.47
7	2.42 ± .07	6.93 ± .63	2.86	1.98 ± .14	5.12 ± .54	2.59	-	-	-
*Aver.	2.37 ± .04	6.25 ± .09	2.64	2.10 ± .04	5.44 ± .10	2.59	2.13 ± .10	5.25 ± .20	2.46

*These averages represent several hundred individual determinations accumulated from seven normal donors over a two-year period. Day 1: n (net flux) = 86; n (exchange flux) = 101. Day 2: n (net flux) = 94; n (exchange flux) = 82. Day 3: n (net flux) = 35; n (exchange flux) = 38.

In all instances the maximal net flux obtained with insulin-treated cells was greater than those of untreated red cells. The average values ± S.E. for the insulin-treated and the untreated cells were respectively 3.45 ± .15 and 2.31 ± .04 ($p < .01$) indicating an average increase of 49% of the maximal net flux by insulin. The values obtained for the exchange flux show a relatively large variation when cells obtained from different donors are utilized and it appears that in certain instances insulin may exert an effect on the exchange flux. However, when all of the data of the individual experiments are summed and averaged, the values ± S.E. for the insulin-treated and control cells are respectively 6.03 ± .21 and 5.73 ± .18 ($p > .10$). There was therefore no significant difference in the exchange flux between insulin-treated and non-treated cells.

The average net and exchange fluxes obtained with non-treated and insulin-treated red cells from nine different donors are shown in Table 3. These values were obtained from both freshly drawn and stored cells. The values obtained for the net flux from the insulin-treated cells when compared to their respective controls show that: 1. The cells of one of the donors (#3) are unresponsive to insulin with respect to glucose transport under maximal net conditions, 2. This unresponsiveness occurs whether the cells are used after 24 hours or when they are freshly drawn, 3. The cells (fresh or stored) of the other donors all show a significant increase in the net flux after being exposed to insulin, 4. The increase in the maximal net efflux ranged from 27 to 81%. The net efflux, excluding the values from the unresponsive donor cells, showed an overall increase of 47%.

The values obtained for the exchange flux from the insulin-treated cells when compared to their respective controls show that: In all cases

TABLE 2. The individual net and exchange fluxes of glucose-C^{14}-UL obtained from insulin-treated and non-treated human red cells. The insulin concentration is in μmoles x 10^{-2} per ml. packed cells. Flux values are expressed as millimoles per liter cell water-sec.

			Net Flux		Exchange Flux	
Donor	Day	Insulin	Control	Insulin Treated	Control	Insulin Treated
1	2	2.7	1.99	2.75	4.86	4.58
			2.08	2.98	4.96	4.77
7	1	13	2.24	4.42	5.68	6.64
				3.56		6.12
2	1	6.7	2.29	2.85	6.07	7.34
			2.46	3.36	6.32	6.69
				3.14		7.84
1	1	13	2.29	2.79	5.66	5.12
			2.45	2.78	5.58	5.58
				2.73		6.70
5	2	13	2.37	3.23	5.75	5.48
			2.29	3.07	6.14	5.23
				3.40		6.14
9	1	26	2.17	2.87	4.75	4.86
				2.93	4.75	5.37
				3.25		
4	1	6.7	2.32	3.04	5.60	5.45
			2.22	3.09	5.33	5.96
6	1	26	2.31	4.49	6.24	6.52
			2.33	4.63	6.62	6.82
8	1	13	2.66	4.60	7.38	7.68
			2.51	4.83		
				4.59		

TABLE 3. The average net and exchange fluxes of glucose-C^{14}-UL obtained from non-treated and insulin-treated red cells. The averaged flux values for individual donors are expressed as millimoles per liter cell water-sec.

Donor	Net Flux ± S.E.				Exchange Flux ± S.E.				E/N	
	Controls	Treated Cells	% Change	p	Controls	Treated Cells	% Change	p	Control	Treated Cells
1(a)	2.32 ± .10	3.27 ± .20	41	<.01	7.05 ± .33	6.52 ± .29	(-8)	>.10	3.04	1.99
1(b)	2.04 ± .08	3.02 ± .08	48	<.01	4.56 ± .22	4.84 ± .34	6	>.10	2.23	1.60
2(a)	2.36 ± .11	3.10 ± .21	32	<.05	6.48 ± .27	5.19 ± .34	(-20)	>.10	2.75	1.67
3(a)	2.41 ± .04	2.90 ± .28	20	>.10	6.03 ± .11	6.50 ± .62	8	>.10	2.50	2.24
3(b)	2.17 ± .05	2.01 ± .08	(-7)	>.10	4.29 ± .09	5.07 ± .16	18	<.01	1.98	2.52
4(a)	2.28 ± .07	3.04 ± .07	33	<.01	-	-	-	-	-	-
5(b)	2.54 ± .07	3.23 ± .08	27	<.01	5.94 ± .20	5.62 ± .27	(-5)	>.10	2.34	1.74
6(a)	2.27 ± .07	4.11 ± .36	81	<.01	6.43 ± .19	6.67 ± .11	4	>.10	2.83	1.51
7(a)	2.42 ± .07	3.33 ± .25	38	<.05	-	-	-	-	-	-
7(b)	1.98 ± .14	2.97 ± .16	50	<.05	6.72 ± 1.09	6.48 ± .33	(-4)	>.10	3.39	2.18
9(a)	2.45 ± .10	4.35 ± .22	78	<.01	-	-	-	-	-	-
10(a)	2.29 ± .05	3.17 ± .08	38	<.01	4.62 ± .25	6.01 ± .25	30	<.05	2.02	1.90

(a) Day 1, freshly drawn cells; (b) Day 2, 24-hour-old cells.

save two the exchange flux was not significantly affected. In both cases the exchange flux of the insulin-treated cells was significantly elevated. It should be noted that the respective controls of these two particular donors are on the very low side of the average exchange flux. In a third case, with donor 2, there is a large decrease in the exchange flux of the insulin-treated cells. However, due to the unusually large fluctuations in the exchange flux values obtained with the cells of this particular donor, this decrease was found not to be statistically significant.

The results with insulin are also reflected in the E/N ratios. Where the net fluxes of insulin-treated cells have been increased, the E/N values of these cells have been markedly reduced. The overall average E/N ratio is 2.56 for the non-treated cells (if the values obtained with donor 3 are omitted the average E/N ratio for non-treated cells is 2.66). The overall average E/N ratio for insulin treated cells is 1.93 and if the values obtained with donor 3 are omitted the average E/N ratio becomes 1.80. This represents an overall decline in the E/N ratio of 25% (32% when the non-responsive donors are omitted).

In a related series of experiments (Table 4), the insulin concentration was extended to a range of from 0.025 to 20 units per ml. packed cells. This corresponds to an approximate range of 0.03×10^{-2} to 0.26 µmoles insulin (M.W. of insulin - 5800) per ml. packed red cells (average hematocrit of 75%). Examination of the exchange fluxes shows that only with the highest concentration of insulin used in this series (26.7×10^{-2} µmoles per ml. cells) was there a significant increase in the exchange flux. No significant effect on the exchange flux could be demonstrated with the other concentrations of insulin. The net fluxes of the insulin-

TABLE 4. The average net and exchange fluxes of glucose-C^{14}-UL obtained from human red cells exposed to different concentrations of bovine insulin for 30 min. at 23-25°C. The insulin concentration is in μmoles x 10^{-2} per ml. packed cells. Fluxes are expressed as millimoles per liter cell water-sec.

Insulin	Net Flux ± S.E.			Exchange Flux ± S.E.			E/N	
	Controls	Treated Cells	p	Controls	Treated Cells	p	Controls	Treated Cells
0.03	2.18 ± .11	2.26 ± .31	>.10	-	-	-	-	-
0.30	2.07 ± .11	2.76 ± .11	<.01	5.99 ± .26	6.55 ± .71	>.10	2.89	2.37
2.7	1.96 ± .09	2.78 ± .09	<.01	6.24 ± .30	6.35 ± .33	>.10	3.18	2.28
6.7	2.20 ± .10	2.84 ± .06	<.01	5.43 ± .24	6.12 ± .32	>.10	2.47	2.15
10.0	1.67 ± .16	2.29 ± .16	<.05	5.35 ± .67	4.83 ± .34	>.10	3.20	2.11
13.3	2.37 ± .07	3.35 ± .16	<.01	6.28 ± .36	5.62 ± .15	>.10	2.65	1.68
26.7	2.28 ± .09	3.17 ± .15	<.01	6.02 ± .16	6.79 ± .23	<.05	2.64	2.14

The values for the insulin concentration in μMoles per ml. packed red cells is based on: 1. An average hematrocrit of 75%, and 2. A molecular weight of 5800 for insulin.

treated cells present a different picture. Here, (with the exception of the value obtained at the lowest concentration of insulin which was utilized) the net flux is significantly elevated at every level of insulin. The threshold level appears to be located between 0.03×10^{-2} and 0.3×10^{-2} μmoles insulin per ml. packed cells. There does not appear to be any linear relationship between the degree of the net flux increase and the level of insulin in the incubation mixture. It should be noted, however, that when the percentage increase of the maximal net efflux at each insulin concentration level is calculated, a 30-40% elevation is almost immediately attained and this average increase is maintained over the entire concentration range. Thus, over a concentration range of 0.30×10^{-2} to 26.7×10^{-2} μmoles insulin per ml. packed cells, the average percentage elevation of the net efflux of glucose is 37% $\pm$ 4%. The E/N values again reflect the elevation of the net flux that occurs in insulin-treated cells. The overall average E/N ratio for the non-treated cells was 2.84 and for the insulin-treated cells it was 2.12.

Experiments utilizing insulin which had been heated in boiling water for 30 minutes resulted in a 80% decrease in the elevation of the maximal net flux normally observed with unheated insulin. Insulin was not heated for longer periods of time since after 30 minutes it tended to become an insoluble gel which could not be quantitatively transferred to the red cell suspension.

Since for the measurement of the maximal net flux, the environment into which the glucose-loaded cells are placed is of a lower osmolarity than the environment in which the red cells are equilibrated with glucose initially, a number of experiments were performed in which insulin-treated

red cells were introduced into a medium devoid of glucose but having an osmolarity adjusted with sodium chloride equal to that in which the red cells were equilibrated. The maximal net flux values of untreated and insulin-treated cells under these conditions were essentially unchanged. An elevation in the maximal net flux of 40% in the insulin-treated red cells was still observed.

In order to further investigate the insulin acceleration of the maximal net flux, red cells from normal donors were incubated as previously described with a variety of different agents. The first group had in common the presence of one disulphide linkage within the molecule. The presence of a disulphide linkage was considered to be important in view of evidence (Fong et al., '62 and Edelman et al., '63) which has suggested that the physiological action of insulin is on the membrane and that the membrane interaction occurs by means of an interchange between the -S-S- bonds of the insulin molecule and the sulfhydryl groups of the target membrane. The first group consisted of oxytocin, vasopressin and oxidized glutathione. As controls for these substances, two compounds lacking disulphide linkages but possessing sulphydryl groups, reduced glutathione and L-cysteine, were included for testing. The levels of these agents as well as the flux values obtained from red cells exposed to these different substances are shown in Table 5. The results show that there is no significant difference between the exchange flux values of non-treated cells and the cells treated with the various agents listed in Table 5. On the other hand, there are significant increases in the net efflux of glucose with those cells which were incubated with either vasopressin or oxidized glutathione. The net flux is not significantly altered in the cells exposed to either oxytocin, reduced glutathione or L-cysteine. The values

TABLE 5. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells treated with agents containing disulfide or sulfhydryl groups. Flux values are expressed as millimoles per liter cell water-sec.

	Net Flux ± S.E.				Exchange Flux ± S.E.				E/N	
	Controls	Treated Cells	% Change	p	Controls	Treated Cells	% Change	p	Control	Treated Cells
Oxytocin	2.40 ± .03	2.61 ± .09	9	<.10	5.87 ± .18	5.27 ± .24	(-10)	>.05	2.44	2.02
Vasopressin	2.36 ± .13	2.89 ± .13	22	<.01	5.31 ± .18	5.86 ± .42	10	>.10	2.25	2.03
GSSG	2.19 ± .03	2.62 ± .07	20	<.01	5.23 ± .27	5.87 ± .52	12	>.10	2.39	2.24
GSH	1.80 ± .21	2.12 ± .08	18	>.10	7.53 ± .68	6.54 ± .98	(-13)	>.10	4.18	3.08
Cysteine	1.96 ± .16	2.24 ± .16	14	>.10	6.62 ± 1.1	6.25 ± .77	(-6)	>.10	3.38	2.79
Insulin	2.28 ± .09	3.17 ± .15	39	<.01	6.02 ± .16	6.79 ± .23	13	>.10	2.64	2.14

The concentrations of the compounds shown above are in μMoles per ml. packed cells and are as follows: oxytocin 0.56; vasopressin 0.54; GSSG 4.08; GSH 7.90; l-cysteine 7.40; insulin 0.28.

obtained from insulin-treated cells are included in Table 5 for comparative purposes.

An additional number of related agents were also examined for their effect on glucose flux in human red cells. They were insulin - A chain, insulin - B chain, insulin-S- sulfo A chain, insulin-S- sulfo B chain, and cysteic acid. The results obtained with these substances are shown in Table 6. The data obtained indicate the following: 1. The net flux is markedly enhanced by the A-chain, B-chain, and to a lesser though still substantial extent by the S- sulfo A-chain and the S- sulfo B-chain. 2. The exchange flux is not significantly increased by any of these agents with the exception of the S- sulfo B chain, 3. Cysteic Acid, used as a representative control for molecules possessing $-SO_3$ groups, gave no significant change in either the net or exchange fluxes.

A third group of agents, lacking disulphide, sulphydryl or $-SO_3$ groups was incubated with red cells and their respective effects on glucose flux examined. These substances and the concentrations used in μmoles per ml packed cells were ACTH (0.47), polymixin B (1.26), poly-glutamic acid (M.W.- 2300 (1.0), 8100 (.28), 11600 (.20)), epinephrine (0.47), and protamine sulphate (.10). The flux values obtained from cells incubated with these materials show the following: 1. Epinephrine, polymixin B, and polyglutamic acid (mol. wt. 8100 and 11,600) have no significant effect on either the net or exchange flux, 2. ACTH and poly-glutamic acid (mol. wt. 2300) significantly increase the net flux by 40% and 20% respectively and have no significant effect on the exchange flux, 3. Protamine sulphate lowers the exchange flux but has no significant effect on the net flux.

Briefly, the results that have been presented so far may be summarized

TABLE 6. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells treated with insulin derivatives. The incubation temperature was 23-25°C. Flux values are expressed as millimoles per liter cell water-sec.

Agent	Net Flux ± S.E.				Exchange Flux ± S.E.				E/N	
	Controls	Treated Cells	% Change	p	Controls	Treated Cells	% Change	p	Controls	Treated Cells
A Chain	2.24 ± .07	3.82 ± .15	70	<.01	6.51 ± .24	6.82 ± .51	5	>.10	2.91	1.79
B Chain	2.26 ± .13	3.51 ± .10	55	<.01	6.42 ± .25	6.70 ± .36	4	>.10	2.84	1.91
S-Sulfo A Chain	2.23 ± .10	2.92 ± .09	31	<.01	6.61 ± .51	6.94 ± .60	13	>.10	2.96	2.38
S-Sulfo B Chain	2.17 ± .08	2.85 ± .13	31	<.01	5.70 ± .48	7.10 ± .26	25	<.05	2.63	2.49
Cysteic Acid	2.21 ± .22	2.24 ± .05	2	>.10	5.72 ± .34	4.41 ± .33	(-23)	>.10	2.59	1.97
Insulin	2.28 ± .09	3.17 ± .15	39	<.01	6.02 ± .16	6.79 ± .23	13	>.10	2.64	2.14

The concentrations of the various agents shown in the above table are expressed in µMoles per ml. packed red cells and are as follows: A chain 0.66; B chain 0.49; S-Sulfo A chain 0.66; S-Sulfo B Chain 0.48; Cysteic acid 8.88; Insulin 0.28.

as follows: 1. The net flux of glucose in the normal non-treated human red cell is of the order of 2.3 millimoles per liter cell water-sec., 2. The exchange flux of glucose in identical cell populations is of the order of 6 millimoles per liter cell water-sec., 3. The ratio E/N is of the order of 2.8, 4. With normal non-treated cells the net flux value remains relatively constant with respect to in vitro storage. The exchange flux on the other hand tends to decrease over the 72 hour period during which the cells are usually stored, and 5. A number of compounds, one of which is insulin, elevate the net flux and with a few exceptions have no effect on the level of the exchange flux.

PCMBS and CHLORMERODRIN

The mediated transfer of glucose across the red cell membrane was further investigated with the use of two sulphydryl inhibitors, namely, p-chlormercuriphenyl sulfonic acid (PCMBS) and chlormerodrin. The structural formulas of these compounds are given in Fig. 14. Red cells were incubated with different quantities of PCMBS for 30 minutes at 22-24°C and the net and exchange fluxes examined as previously described. The results which were obtained are shown in Table 7. It should be noted here that PCMBS does not penetrate the red cell membrane (van Steveninck et al., '65) and accordingly it is believed that its action is accomplished by its binding to a relatively few SH groups located on the surface of the membrane. The results show the following: 1. At the lowest concentration of PCMBS (0.05 μmoles per ml. packed cells) which was utilized, the exchange flux is considerably inhibited (51%), while the net flux is unaffected, 2. Over the concentration range (0.05-7.8 μmoles per ml. packed cells) of PCMBS which was employed the percentage inhibition of the exchange flux ranged from a low of 41% to a high of 73%. As a result, the exchange flux of the PCMBS-treated cells was reduced to the

TABLE 7. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells exposed to different concentrations of p-chloromercuriphenylsulfonic acid (PCMBS). Red cells were incubated with PCMBS for 30 min. at 22-24°C. The PCMBS concentration is expressed as µmoles per ml. packed cells. Flux values are expressed as millimoles per liter cell water-sec.

PCMBS	Net Flux ± S.E.			Exchange Flux ± S.E.			E/N	
	Controls	Treated Cells	% Inhib.	Controls	Treated Cells	% Inhib.	Controls	Treated Cells
0.05	2.59 ± .12	2.41 ± .26	7	6.63 ± .25	3.27 ± .43	51	2.56	1.36
0.35	2.13 ± .23	1.95 ± .18	8	6.22 ± .16	3.70 ± .24	41	2.92	1.90
0.7	1.64 ± .05	1.44 ± .14	12	7.28 ± .58	2.87 ± .92	61	4.43	1.99
1.4	2.09 ± .15	1.71 ± .10	18	5.26 ± .21	2.82 _ .25	46	2.52	1.65
1.9	2.33 ± .06	1.81 ± .08	22	6.13 ± .21	2.16 ± .22	65	2.63	1.19
3.8	2.23 ± .14	1.53 ± .11	31	5.85 ± .54	1.56 ± .19	73	2.62	1.02
7.8	1.86 ± .17	1.18 ± .12	37	5.54 ± .31	1.78 ± .31	68	2.98	1.51

There was no significant difference ($p > .10$) between the net flux values obtained with the three lowest PCMBS concentrations and their respective controls.

level of the normal net flux value, 3. By comparison, the net flux of glucose was not significantly inhibited until the cells were exposed to a PCMBS concentration of 1.4 umoles per ml. packed cells. This concentration is approximately midway in the range of the PCMBS concentrations which were utilized. In addition, it should be noted that at this level of PCMBS, where the net flux is inhibited by 18%, the exchange flux in the same cell population is inhibited to the extent of 46%. Conversely, when the exchange flux is inhibited to 40-50% at very low PCMBS concentrations the net flux is unaffected. The use of greater quantities of PCMBS was not possible in that the cells clumped, making accurate sampling impossible.

Red cells were similarly treated with different concentrations of chlormerodrin. The cells were incubated with this inhibitor both at 22-24°C and at 4°C. The incubation time was 30 minutes. It has been reported (van Steveninck et al., '65) that at 4°C chlormerodrin does not penetrate the red cell membrane or at least does so extremely slowly, while at 24°C it penetrates at a relatively higher rate. The flux data obtained from cells treated with chlormerodrin at 22-24°C are shown in Table 8. It can be seen that chlormerodrin inhibits both the net and exchange flux equally well and that the degree of inhibition in both cases increases with increased chlormerodrin levels. At chlormerodrin concentrations greater than 0.55 μmoles per ml. red cells, the exchange flux was reduced to the level of the net flux obtained with normal non-treated cells. At the highest chlormerodrin concentration, both the net and the exchange flux were almost totally inhibited. The E/N ratios for the chlormerodrin-treated cells reflect the almost identical degree of inhibition of both fluxes and as such stay relatively constant and closer to an average value of 2.3 by comparison to the E/N values obtained from

TABLE 8. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells exposed to different concentrations of chlormerodrin. Red cells were incubated with chlormerodrin for 30 min. at 22-24°C and at 4°C. Chlormerodrin concentrations are expressed as μmoles per ml. packed cells. Flux values are expressed as millimoles per liter cell water-sec.

Temp.	Initial Conc.	Final Conc.*	Net Flux ± S.E.			Exchange Flux ± S.E.			E/N	
			Controls	Treated Cells	% Inhib.	Controls	Treated Cells	% Inhib.	Controls	Treated Cells
22-24°C	0.14	.08	2.62 ± .20	2.19 ± .15	16	6.13 ± .43	5.02 ± .26	18	2.34	2.29
	0.27	.15	2.28 ± .17	1.76 ± .08	23	5.19 ± .33	3.48 ± .22	33	2.28	1.98
	0.55	.30	2.19 ± .10	1.08 ± .08	51	5.84 ± .27	2.52 ± .24	57	2.67	2.33
	1.1	.59	2.31 ± .13	0.57 ± .38	75	5.79 ± .39	1.57 ± .30	73	2.51	2.75
	2.7	1.5	2.03 ± .10	0.51 ± .06	75	4.94 ± .70	0.99 ± .20	80	2.43	1.94
4°C	0.14	.14	2.54 ± .12	1.31 ± .05	49	5.90 ± .22	3.98 ± .25	33	2.32	3.04
	0.54	.54	2.88 ± .16	1.47 ± .15	49	5.78 ± .22	2.35 ± .21	59	2.01	1.60

The values for the net and exchange fluxes obtained from chlormerodrin-treated cells were all significantly different from their respective controls. *These values represent the level of chlormerodrin calculated to be present in the cell water or on the outer surface of the red cell after a 30 minute incubation period.

PCMBS-treated cells (Table 7). The values under "Final Conc." in Table 8. are corrected for the passage at 24°C of chlormerodrin through the cell and represent chlormerodrin levels which are found in the cell water and on the outer cell surface after 30 minutes incubation. When these concentrations are utilized for comparative purposes it can be seen that the exchange and maximal net fluxes are inhibited to a greater extent at 24°C than at 4°C by chlormerodrin.

As a result of the opposite effects obtained with PCMBS, chlormerodrin and insulin, experiments were conducted where both mercurial and insulin were present. Red cells were first incubated with insulin, followed by the addition of the inhibitor, or cells were incubated first with the particular inhibitor after which insulin was added, or, as in the case with PCMBS, cells were incubated simultaneously with inhibitor and insulin. In the experiments with PCMBS, the temperature was always in the range of 22-24°C and the incubation time with PCMBS and insulin was 30 minutes for each. The concentration of PCMBS was that which consistently gave 70-80% of the maximum inhibition that could normally be obtained. The insulin concentration was adjusted to always be 0.14 μmoles per ml. packed red cells. The results obtained with PCMBS in combination with insulin are shown in Table 9. They show that at the concentration of PCMBS used, the net flux is inhibited by 22%. If PCMBS-inhibited cells are now exposed to insulin, the inhibition falls to 12%. Since this new value, 1.99 $\pm$ .10 millimoles per liter cell water-sec., is not significantly different ($p > .10$) from the net flux of the non-treated cells, the relief of the inhibition of the net flux by insulin could be considered to be 100%. When insulin-treated cells are exposed to PCMBS, the net flux (2.36 $\pm$ .15 millimoles per liter cell water-sec.) is not significantly different ($p > .10$) from the flux obtained with the control (2.25 $\pm$ .07) and therefore the inhibition of the net flux by PCMBS is prevented. When red cells are

TABLE 9. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells. Measurements were made on: 1. Cells incubated with PCMBS for 30 min., 2. Cells incubated with PCMBS for 30 min. followed by insulin for 30 min., 3. Cells incubated with insulin for 30 min. followed by PCMBS for 30 min., 4. Cells exposed to PCMBS and insulin simultaneously for 30 min. The incubations took place at 22-24°C. Fluxes are expressed as millimoles per liter cell water-sec.

	Net Flux ± S.E.	% Inhib.	*p	Exchange Flux ± S.E.	% Inhib.	*p	E/N
Control (untreated)	2.25 ± .07	-	-	6.10 ± .19	-	-	2.71
PCMBS-Treated Cells	1.76 ± .07	22	-	2.11 ± .20	65	-	1.20
PCMBS-Treated Cells Exposed to Insulin	1.99 ± .10	12	>.10	3.93 ± .24	36	<.01	1.97
Insulin-Treated Cells Exposed to PCMBS	2.36 ± .15	0	<.01	4.04 ± .32	34	<.01	1.71
Cells Treated with PCMBS and Insulin Simultaneously	2.23 ± .15	0	<.05	3.70 ± .91	39	<.05	1.66

*The values for p represent the presence or absence of significant differences between values obtained from the PCMBS-treated cells and the values obtained when insulin is present.

exposed to both PCMBS and insulin simultaneously, there is no inhibition of the net flux.

The exchange flux of red cells exposed only to PCMBS was reduced by 65% bringing the exchange flux to a value lower than that of the net flux of normal untreated cells. When insulin is added to the cells after PCMBS, the inhibition of the exchange flux is 36%, a recovery of 45%. When insulin is added prior to the addition of PCMBS, the inhibition of the exchange flux is decreased by half to 34%, and when the cells are incubated with both PCMBS and insulin simultaneously, the inhibition of the exchange flux is 39%, a recovery of 40%. However, unlike the results with the net flux, the inhibition of the exchange flux is never completely prevented or reversed. At the least, the net component is returned to its normal level and at the most the inhibition of the exchange flux component is slightly relieved.

A similar group of experiments were run with chlormerodrin in combination with insulin. Due to the fact that the permeability to chlormerodrin is temperature-dependent, the experiments were performed both at 4°C and at 22-24°C. The data from these experiments is shown in Tables 10 and 11. It can be seen from the data in Table 10 that at 4°C insulin has no effect on the extent of inhibition of the net flux by chlormerodrin. The net flux is inhibited by slightly less than 50% and whether insulin is added before or after the addition of chlormerodrin, the level of inhibition remains essentially the same as with chlormerodrin alone. On the other hand, the exchange flux which is inhibited by 61% shows a significant and considerable recovery in the presence of insulin. When chlormerodrin-treated cells are exposed to insulin the inhibition is 31%, a recovery of 50%, and when insulin-treated cells are exposed to chlormerodrin the inhibition is 23%, a recovery of over 60%.

TABLE 10. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells. Red cells were exposed to: 1. Chlormerodrin only, 2. Chlormerodrin followed by insulin, and 3. Insulin followed by chlormerodrin. The incubation time for each agent was 30 min. and the incubation temperature was 4°C. Fluxes are expressed as millimoles per liter cell water-sec.

	Net Flux ± S.E.	% Inhib.	*p	Exchange Flux ± S.E.	% Inhib.	*p	E/N
Control (untreated cells)	2.70 ± .16	-	-	5.67 ± .29	-	-	2.10
Chlormerodrin-Treated Cells	1.37 ± .16	49	-	2.21 ± .19	61	-	1.61
Chlormerodrin-Treated Cells Exposed to Insulin	1.52 ± .17	44	>.10	3.91 ± .45	31	<.01	2.57
Insulin-Treated Cells Exposed to Chlormerodrin	1.52 ± .14	44	>.10	4.37 ± .56	23	<.01	2.88

*The values for 'p' represent the presence or absence of significant differences between values obtained from the chlormerodrin-treated cells and the values obtained when insulin is present.

TABLE II. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells. Red cells were exposed to: 1. Chlormerodrin only, 2. Chlormerodrin followed by insulin, and 3. Insulin followed by chlormerodrin. The incubation time for each agent was 30 min. and the incubation temperature was 22-24°C. Fluxes are expressed as millimoles per liter cell water-sec.

	Net Flux ± S.E.	% Inhib.	*p	Exchange Flux ± S.E.	% Inhib.	*p	E/N
Control (untreated cells)	2.28 ± .10	-	-	6.29 ± .21	-	-	2.76
Chlormerodrin-Treated Cells	1.18 ± .12	48	-	2.79 ± .32	56	-	2.36
Chlormerodrin-Treated Cells Exposed to Insulin	1.70 ± .13	25	$< .05$	3.14 ± .46	50	$> .10$	1.85
Insulin-Treated Cells Exposed to Chlormerodrin	1.23 ± .17	46	$> .10$	3.19 ± .75	49	$> .10$	2.59

*The values for 'p' represent the presence or absence of significant differences between values obtained from the chlormerodrin-treated cells and the values obtained when insulin is present.

At 24°C (Table II) the exchange flux is inhibited by chlormerodrin to the extent of 56% and this inhibition is not significantly altered by the presence of insulin whether added after chlormerodrin incubation (50% inhibition) or prior to chlormerodrin incubation (49% inhibition). At 24°C the net flux is inhibited by chlormerodrin to the extent of 48%. This inhibition is not significantly altered by the presence of insulin prior to the addition of chlormerodrin. The degree of inhibition is however significantly lessened by almost 50% when insulin is added to red cells which have been exposed to chlormerodrin.

In summary, the results obtained from cells treated with the chlormerodrin-insulin combinations at 4°C and at 24°C indicate the following:

1. At 4°C, a temperature at which for all practical purposes chlormerodrin may be considered as a non-penetrant, and regardless of the order of incubation with insulin, the inhibition of the net flux is unaltered, while the inhibition of the exchange flux is considerably decreased.
2. At 22-24°C, a temperature at which chlormerodrin slowly penetrates the red cell membrane, and regardless of the order of incubation with insulin, the degree of inhibition of the exchange flux is unaltered. In the case where insulin is added first, the degree of inhibition of the net flux is unaltered while in the case where the insulin is added last, the degree of inhibition of the net flux is considerably lessened.

Phospholipases

The inhibition of the exchange flux of glucose and not of the net efflux in human red cells by phospholipase C has been reported (Odesser and Mawe, '67). This area was further investigated and in addition, experiments were carried out which involved the combined use of insulin and phospholipase C (Phl-C).

Red cells were exposed in a number of experiments to different concentrations of phospholipase C solutions for short periods of time (10-15 min.) and glucose fluxes were measured under net and exchange conditions. The results of these experiments are shown in Table 12. The data indicate that over the range of Phl-C concentrations tested (0.104-0.20 mg per ml. packed red cells) there is no significant effect on the net efflux. At the 0.1 and 0.2 mg levels there is a signficant inhibition of the exchange flux. A previous report (Odesser and Mawe, '67), has shown exchange flux inhibitions of greater than 80%. However, in these instances the concentration of the phospholipase was much greater. At these higher concentrations, a considerable degree of hemolysis (>60%) is evident. In a separate series of experiments, the effect of the hemolysate on glucose transport was examined and found to be absent. Even though the hemolysate had no effect on glucose flux, it was considered desirable in the present series of experiments to maintain the level of hemolysis as low as possible. Therefore, the concentration of Phl-C was controlled so that hemolysis never exceeded 30%, while still allowing the demonstration of inhibition of the exchange flux of glucose transport.

Since insulin had relieved the inhibition caused by PCMBS and chlormerodrin under some conditions as previously shown, experiments were carried out in a similar fashion with insulin and Phl-C. The results of these experiments are shown in Table 13. They show that there is no inhibition of the net flux by Phl-C. In those cases where insulin is also present, the net flux is significantly increased above the net flux value obtained with either non-treated or Phl-C-treated cells. In this series of experiments the exchange flux of cells treated with Phl-C was inhibited by 31%. When insulin-treated cells were exposed to Phl-C there was essen-

TABLE 12. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells exposed to different concentrations of phospholipase C (Phl-C). Phl-C concentrations are in mg. per ml. packed cells. Fluxes are expressed as millimoles per liter cell water-sec.

Phl-C	Net Flux ± S.E.				Exchange Flux ± S.E.				E/N	
	Control	Treated Cells	% Inhib.	p	Control	Treated Cells	% Inhib.	p	Control	Treated Cells
0.04	2.26 ± .14	2.09 ± .19	7	>.10	5.85 ± .44	5.10 ± .19	13	>.10	2.59	2.44
0.10	2.54 ± .08	2.41 ± .08	5	>.10	6.07 ± .31	4.16 ± .38	32	<.01	2.39	1.73
0.20	2.31 ± .06	2.48 ± .09	0	>.10	5.97 ± .35	4.15 ± .17	31	>.01	2.58	1.67

TABLE 13. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells exposed to: 1. Phospholipase C, 2. Insulin followed by phospholipase C, 3. Phospholipase C followed by insulin. Fluxes are expressed as millimoles per liter cell water-sec.

	Net Flux ± S.E.	% Change	*p	Exchange Flux ± S.E.	% Change	*p	E/N
Untreated Cells	2.38 ± .06	-	-	6.16 ± .34	-	-	2.59
Phl-C-Treated Cells	2.38 ± .08	0	-	4.24 ± .26	-31	<.01	1.78
Insulin-Treated Cells Exposed to Phl-C	2.97 ± .14	+25	<.01	5.70 ± .17	- 7	>.10	1.92
Phl-C-Treated Cells Exposed to Insulin	2.67 ± .10	+12	<.05	4.73 ± .14	-23	<.01	1.77

*The values for 'p' in the above table are for comparisons between the treated samples and their respective controls. There is no significant difference (p >.10) between the exchange flux values of 4.24 and 4.73.

tially no inhibition of the exchange flux. As can be seen, there is no significant difference ($p > .10$) between the exchange fluxes of non-treated cells ($6.16 \pm .34$) and the insulin-treated cells exposed to Phl-C ($5.70 \pm .17$). On the other hand, the addition of insulin to red cells already exposed to Phl-C did not significantly alter the degree of inhibition of the exchange flux caused by the enzyme. In one experiment (not shown in Table 13) insulin and Phl-C were mixed together and the mixture was added to a suspension of red cells. In this experiment, the exchange flux was inhibited by 25% while the net flux showed a small increase (15%).

In the above experiments notice was taken of the fact that the extent of hemolysis caused by Phl-C was less when the cells had been first exposed to insulin. The extent of this apparent protection was investigated. The red cells were obtained and washed with 0.075 M glucose-saline-phosphate as previously described. Non-treated red cells and insulin-treated red cells (0.072 μmoles insulin per ml. packed red cells) were exposed to different quantities of Phl-C for 20 minutes and the degree of hemolysis was determined. The results presented in Table 14 show that insulin pre-treatment results in a significant decrease in the extent of the hemolysis caused by Phl-C.

The action of several other phospholipases on the transport of glucose was also examined. The results obtained from red cells exposed to Phl-A, Phl-B, Phl-D and cottonmouth venom are shown in Table 15. The results show that at the concentrations used, the net efflux was not significantly altered while the exchange flux was significantly inhibited by all the enzymes with the exception of Phl-B. Where it did occur, hemolysis was never greater than 10%.

TABLE 14. The hemolysis of human red cells with phospholipase C in the presence of insulin. Phl-C concentrations are in mg. per ml. packed cells.

Phl-C	% Lysis: Non-Treated Cells	% Lysis: Insulin*-Treated Cells	% Inhibition of Lysis
0.005	0	0	-
0.010	1.3	0.2	-
0.025	7.2	2.5	65
0.050	32.2	12.3	62
0.100	71.2	46.2	35

*The insulin concentration was 0.072 μMoles per ml. packed red cells.

TABLE 15. The average net and exchange fluxes of glucose-C^{14}-UL in human red cells exposed to a variety of phospholipases. Fluxes are expressed in millimoles per liter cell water-sec.

Agent	Net Flux ± S.E.				Exchange Flux ± S.E.				Exchange Flux / Net Flux	
	Control	Treated Cells	% Inhib.	p	Control	Treated Cells	% Inhib.	p	Control	Treated Cells
Phl-A	2.73 ± .10	2.67 ± .12	2	>.10	7.86 ± .42	5.21 ± .87	34	<.05	2.88	1.95
Phl-B	2.21 ± .07	1.98 ± .16	10	>.10	5.71 ± .68	4.95 ± .62	13	>.10	2.58	2.50
Phl-D	2.21 ± .12	1.98 ± .08	10	>.10	5.67 ± .32	4.69 ± .39	17	<.10	2.57	2.37
Cottonmouth Venom	2.31 ± .17	1.95 ± .16	15	>.10	5.83 ± .67	4.05 ± .52	31	<.10	2.53	2.07

The concentrations of the above agents in mg. per ml. packed red cells were as follows: Phl-A 2.0; Phl-B 1.0; Phl-D 3.0; Cottonmouth venom 0.1.

Binding Experiments

The effects on the exchange and net fluxes caused by insulin, PCMBS, and chlormerodrin, both singly and in combination, were examined. A series of experiments were undertaken in which the degree of binding to the red cell was determined for each of the agents. The binding was measured under the same conditions that exist during the flux measurements. The concentrations of the agents being investigated were comparable to the concentrations of these agents when used in the flux experiments. The cells were obtained and washed as previously described. Glucose-loaded cells were used in all of the binding experiments.

In the first group, red cells were incubated with different concentrations of PCMBS-Hg^{203} at 22-24°C for 30- or 60-minute periods. The cells were then washed five times with either a saline phosphate solution at pH 7.4 or a saline phosphate solution at pH 7.4 containing 0.075 M. glucose. The choice of wash solution did not affect the final result. The amount of labeled PCMBS in the wash solutions was counted and the last wash contained less then 0.05% of the label added initially. Red cells were treated in a similar fashion with chlormerodrin-Hg^{203}. In addition to the different concentrations and incubation periods, some experiments with chlormerodrin were run at 4°C. The results with PCMBS are shown in Table 16 and those obtained with chlormerodrin in Table 17. The number of cells per ml. of packed red cells with a hematocrit of 80-85% is taken as 1×10^{10} (Guyton, '66). The surface area of the red cell used here is 145×10^{8} Å (Westerman et al., '61). These values are used to calculate the approximate number of molecules that are bound per cell as well as per 1000 Å of surface area. The data from both groups indicate an approximate linear relationship between the µmoles added and the

TABLE 16. The binding of PCMBS-Hg^{203} to human red cells. Red cells were incubated with labeled PCMBS for 30 min. at 22-24°C.

Exp.	μMoles PCMBS Added per ml. Packed Cells	μMoles PCMBS Bound per ml. Packed Cells	Molecules PCMBS $\times 10^8$ per Cell	Molecules PCMBS per 10^3 $Å^2$ Surface Area
1	0.35	0.25	0.15	1
	0.35	0.25	0.15	
2	1.89	1.05	0.63	4.5
*3	1.89	1.59	0.96	6.8
	1.89	1.56	0.94	
4	3.77	2.45	1.48	10
	3.77	2.43	1.46	
*5	3.77	2.88	2.07	15

*In these experiments the cells were incubated with PCMBS for 60 minutes. The hematocrits in the different experiments are as follows: #1 84%; #2 78%; #3 80%; #4 84%; #5 70%.

TABLE 17. The binding of chlormerodrin-Hg^{203} to human red cells.

Exp.	°C	μMoles Chlormerodrin Added per ml. Packed Red Cells	μMoles Chlormerodrin Bound per ml. Packed Red Cells	Molecules Chlormerodrin x 10^8 per Cell	Chlormerodrin Molecules per $10^3 Å^2$ Surface Area
1	22-24	0.136	0.094	0.056	0.4
		0.136	0.093	0.056	
2	22-24	0.54	0.50	0.3	2
3	22-24	1.09	0.96	0.58	4
		1.09	0.97	0.58	
4	22-24	1.09	0.97	0.69	5
A	4	0.55	0.27	0.19	1.4
		0.55	0.27	0.19	
*B	4	0.55	0.45	0.31	2.2
		0.55	0.43	0.31	

*In this experiment the cells were incubated with chlormerodrin for 60 minutes. In all the other experiments the incubation time with chlormerodrin was 30 minutes. The hematocrits in the different experiments are as follows: #1 80%; #2 78%; #3 80%; #4 70%; A 74%; B 74%.

number of μmoles bound. With the same chlormerodrin concentrations there is less bound in 30 minutes at 4°C than at 24°C.

The binding of Insulin-I^{131} to red cells was determined in a manner similar to that described above. The red cells were incubated with different concentrations of Insulin-I^{131} for various time periods after which the cells were washed with either the glucose-saline phosphate solution or saline phosphate solution previously described. The data obtained with different insulin concentrations are shown in Table 18. From the data it appears that the number of μmoles of insulin bound shows an approximately linear relationship to the number of moles of insulin added initially. In general, 1-2% of the insulin added initially remains bound to the red cells.

A number of experiments were carried out where the effect of insulin on both PCMBS and chlormerodrin binding was investigated. In these experiments the cells were either incubated first with cold insulin and then exposed to the labeled inhibitor or were first exposed to the labeled inhibitor and then to insulin. The concentrations of insulin, PCMBS, and chlormerodrin used in these experiments were comparable to the concentrations used in determining the net and exchange fluxes. The experiments with chlormerodrin were carried out at both 24 and 4°C. The results of these experiments are presented in Tables 19 and 20. It is readily apparent that regardless of the order of addition, insulin (at the concentration used) had no effect on the amount of either PCMBS or chlormerodrin that bound to the red cells.

TABLE 18. The binding of insulin-I[131] to human red cells. Red cells were incubated with labeled insulin for 30 min. at 23-25°C.

Donor	Wash*	μMoles Insulin Added per ml. Packed Cells	μMoles Insulin Bound per ml. Packed Cells	# of Molecules of Insulin Bound per 10^6 Å^2 Red Cell Surface Area	
				(1) Theoretical Maximum	(2) Experimentally Found
6	GSP	2.8×10^{-8}	0.15×10^{-8}	1.16×10^{-4}	6.5×10^{-6}
6	SP	"	0.10×10^{-8}	"	4.3×10^{-6}
1	GSP	"	0.09×10^{-8}	"	4.1×10^{-6}
1	SP	"	0.10×10^{-8}	"	4.3×10^{-6}
2	GSP	"	0.16×10^{-8}	"	7.0×10^{-6}
2	SP	"	0.18×10^{-8}	"	8.0×10^{-6}
1	GSP	3.1×10^{-3}	0.29×10^{-4}	13.3	0.13
1	SP	"	0.36×10^{-4}	"	0.16
2	GSP	"	0.30×10^{-4}	"	0.13
2	SP	"	0.36×10^{-4}	"	0.16
1	GSP	2.8×10^{-2}	0.12×10^{-3}	230	1.0
1	SP	"	0.14×10^{-3}	"	1.2
1	GSP	2.8×10^{-1}	0.27×10^{-2}	2300	24.0
1	SP	"	0.22×10^{-2}	"	24.4

*Exchange conditions are maintained when the cells are washed with glucose-saline-phosphate (GSP) and maximal net conditions are maintained when the cells are washed with saline-phosphate (SP).

TABLE 19. The binding of PCMBS-Hg^{203} to human red cells in the presence of insulin. Insulin is added either prior to or following the incubation with PCMBS. All incubations were at 22-24°C. Unless otherwise noted the incubation time with PCMBS was 30 min.

Experiment no.	1	2	3
μMoles Insulin per ml. Cells	0.14×10^{-4}	0.28×10^{-4}	0.28×10^{-4}
μMoles PCMBS Added	3.77	1.89	1.89
	μMoles PCMBS Bound per ml. Packed Red Cells ± S.E.		
PCMBS-Treated Cells	2.88 ± .08	1.05 ± .04	1.58 ± .03
PCMBS-Treated Cells Followed by Insulin	3.19 ± .06*		1.52 ± .02
Insulin-Treated Cells Followed by PCMBS	2.70 ± .03	1.14 ± .04	

*Exposed to PCMBS for a total time of 60 minutes.

TABLE 20. The binding of chlormerodrin-Hg^{203} to human red cells in the presence of insulin. Insulin is added either prior to or following the incubation with chlormerodrin. Unless otherwise noted the incubation time with chlormerodrin was 30 minutes.

Experiment no.	1	2	3	4
μMoles Insulin per ml. Cells	0.14×10^{-4}	0.28×10^{-4}	0.28×10^{-4}	0.28×10^{-4}
μMoles Chlormerodrin Added	1.09	0.55	0.55	0.55
Incubation Temperature °C	22	22	4	4
	μMoles Chlormerodrin Bound per ml. Packed Red Cells ± S.E.			
Chlormerodrin-Treated Cells	0.97 ± .01	0.50 ± .02	0.10 ± .01	0.44 ± .01
Chlormerodrin-Treated Cells Exposed to Insulin	0.92 ± .03*	0.49 ± .01*	-	0.41 ± .01*
Insulin Treated Cells Exposed to Chlormerodrin	0.96 ± .01	0.49 ± .01	0.09 ± .01	-

*Exposed to chlormerodrin for a total time of 60 minutes.

The aspect dealing with binding was concluded with a group of experiments in which the effect of PCMBS and chlormerodrin on insulin binding was investigated. The cells were exposed to the sulphydryl inhibitors either prior to or following incubation of the red cells with insulin-I^{131}. The experiments with insulin and PCMBS were carried out at 22-24°C. As in the other binding experiments, the various concentrations are comparable to those used in the flux experiments. Two concentrations of PCMBS were utilized. The lower concentration is one which does not cause inhibition of the net flux but which does inhibit the exchange flux (Table 7). At the higher concentration the net flux is lowered by approximately 20% while the exchange flux is reduced to a value even below that of the normal net flux. The results obtained in the PCMBS-insulin experiments are shown in Table 21. These show that the addition of PCMBS to the cells after insulin incubation, has no effect on the amount of insulin bound to the cells. However, when insulin is added to PCMBS treated cells, there is a marked decrease (64-68%) in the amount of insulin which will bind to the cells. This decrease occurs to the same extent at both PCMBS levels.

The results obtained with chlormerodrin and labeled insulin are shown in Table 22. At 4°C, the presence of chlormerodrin (regardless of the order of addition) reduces the amount of insulin which binds to the cells by 41-45%. In a similar manner, at 22-24°C, less insulin binds to the cells in the presence of chlormerodrin. However, at this temperature the pre-incubation of the cells with chlormerodrin effects a 100% greater inhibition of insulin binding than when chlormerodrin is added to the cells after they have been exposed to insulin.

TABLE 21. The binding of Insulin-I[131] to human red cells in the presence of PCMBS. The reactions were run at 22-24°C. The PCMBS concentrations were 0.05 and 1.89 μmoles per ml. cells. Insulin was added to give an initial concentration of 3.1×10^{-3} μmoles per ml. red cells.

	Insulin, μMoles x 10 ± S.E. Bound per ml. Red Cells	% Change	p
Insulin-Treated Cells	0.73 ± .10	-	-
Insulin-Treated Cells Exposed to PCMBS (0.05 μmoles per ml. Cells)	0.58 ± .08	(-20)	$>$ .10
Insulin-Treated Cells Exposed to PCMBS (1.89) μmoles per ml. Cells)	0.95 ± .27	30	$>$ .10
PCMBS (0.05 μmoles per ml. Cells)-Treated Cells Exposed to Insulin	0.23 ± .06	(-68)	$<$ .05
PCMBS (1.89 μmoles per ml. Cells)-Treated Cells Exposed to Insulin	0.26 ± .04	(-64)	$<$ .05

TABLE 22. The binding of Insulin-I^{131} to human red cells in the presence of chlormerodrin. The reactions were run at 22 and at 4°C. The chlormerodrin concentration was 0.54 µmoles per ml. packed cells. Insulin was added to give an initial concentration of 3.1×10^{-3} µmoles per ml. packed cells.

°C		Insulin, µMoles $\times 10^{-4} \pm$ S.E., Bound per ml. Red Cells	% Change	p
22	Insulin-Treated Cells	0.60 ± .06	-	-
	Insulin-Treated Cells Exposed to Chlormerodrin	0.43 ± .01	(-30)	<.05
	Chlormerodrin-Treated Cells Exposed to Insulin	0.22 ± .04	(-64)	<.01
4	Insulin-Treated Cells	0.22 ± .01	-	-
	Insulin-Treated Cells Exposed to Chlormerodrin	0.13 ± .01	(-41)	<.01
	Chlormerodrin-Treated Cells Exposed to Insulin	0.12 ± .01	(-45)	<.01

Diabetic Donors

The following experiments were a direct outgrowth of the *in vitro* insulin experiments already mentioned. The following data obtained from the red cells of diabetic human donors represent preliminary results which, while they do not appear to shed any light on the mechanism of sugar transport, may prove useful in the clinical studies.

For these experiments, red cells were obtained from several male subjects, diagnosed as diabetics, who were either on injectable insulin therapy or on a diet-controlled regimen. None of these individuals had received oral insulin substitutes prior to the time at which blood samples were taken. Red cells were normally obtained in the morning, and in those cases where the donors were on insulin therapy, the red cells were usually obtained approximately one hour after injection. The cells were washed as previously described and where possible flux values were obtained on both day 1 and day 2. In a few instances, insulin was added *in vitro* to the cells for the usual incubation period and the values thus obtained are shown in brackets. Most of the data however, are from control (non-*in vitro* insulin-treated) red cells.

The individual net and exchange flux values obtained with red cells from diabetic subjects are shown in Table 23. When these results are compared with those obtained from normal donors (Table 1), it is readily apparent that the net fluxes obtained from this group of diabetic subjects are in every case substantially higher than in non-treated red cells obtained from non-diabetic (normal donors). In fact, a number of individual values are by themselves nearly as great as the highest fluxes obtained with insulin-treated normal cells (Table 2). In those instances where insulin was added *in vitro*, there appeared to be no additional

TABLE 23. The net and exchange fluxes of glucose-C^{14}-UL in red cells obtained from male donors diagnosed as diabetic. Flux values are expressed as millimoles per liter cell water-sec.

Donor	Day 1 Net Flux	Day 1 Exchange Flux	Day 2 Net Flux	Day 2 Exchange Flux
MA	3.02 [2.70]	5.67	-	-
	2.57 [2.89]	5.28		
	2.67			
NP	3.22	7.62	2.67 [2.99]	5.54
	4.20	5.77	4.22 [2.82]	5.98
JB	3.51	9.73	-	-
	3.38			
CW	2.75	8.55	2.61	5.87
	2.75	5.13	2.02	
AB	4.06	9.19	2.85	5.87
	3.97	10.15	3.02	6.09
CM	2.60	5.88	2.73	4.13
	2.92	6.03	2.00	4.35
*HE	2.83 [4.84]	6.74	1.97	3.12
	3.45 [3.93]	6.12	1.76	3.56
*FE	4.25 [3.75]	7.28	2.09 [2.17]	4.37 [5.22]
	2.78 [2.85]	5.96	2.47 [2.62]	5.53 [4.42]

*These donors are on 'diet control' therapy. All the other donors receive protamine insulin. The values in brackets were obtained with washed diabetic donor red cells which were afterwards incubated with bovine insulin *in vitro* as described in Materials and Methods. All the other values are derived from washed diabetic donor red cells which are not exposed to insulin after their removal from their donor.

increase in the net flux values over the non-treated samples. The exchange fluxes obtained with the red cells of this group of donors remain with the exception of a few individual values, well within the normal spread. The decline in the exchange flux and the net flux from day 1 to day 2 is similar in degree to that obtained with normal cells.

The net and exchange flux of the red cells from two diabetic subjects on diet control therapy were obtained and are also shown in Table 23. The initial (day 1) net fluxes are high and on the average they are slightly raised with the *in vitro* addition of insulin. Unlike the behavior of the net flux of the cells of the first group of diabetic donors, the net flux values decline dramatically after 24 hours to the level obtained with non-treated cells from normal donors. Addition of insulin *in vitro* to these cells after 24 hours storage does not significantly alter the net flux. The exchange fluxes obtained with the cells from the diet-controlled diabetic donors is essentially similar to values obtained with untreated cells from normal donors. However, like the net flux obtained with the cells of these particular subjects, the exchange flux obtained from cells after they have been stored for 24 hours shows a dramatic decline. Addition of insulin *in vitro* to these cells, has no significant effect on the exchange flux. In Table 24 the results obtained with these two groups of donors are compiled and their averages are compared with the values obtained from untreated normal red cells. These results show that: 1. The net flux on day 1 obtained from cells of diabetics on insulin therapy is greater than the control value by 35% and the net flux of the diet controlled subjects exceeds the net flux of the control by 40%, 2. The exchange flux values on day 1 of both sets of diabetic donors and the controls are not significantly different,

TABLE 24. A comparison of the net and exchange fluxes of glucose-C^{14}-UL in non-treated red cells obtained from normal, diabetic (insulin therapy), and diabetic (diet-controlled) donors. Fluxes are expressed as millimoles per liter cell water-sec.

Day 1 Donors	Net Flux ± S.E.	% Change	p	Exchange Flux ± S.E.	% Change	p	E/N
Normal	2.37 ± .04	-	-	6.25 ± .09	-	-	2.64
Diabetic (Insulin)	3.21 ± .15	35	<.01	7.12 ± .54	9	>.10	2.22
Diabetic (Diet Control)	3.32 ± .34	40	<.05	6.53 ± .30	0	>.10	1.97

Day 2 Donors	Net Flux ± S.E.	% Change	p	Exchange Flux ± S.E.	Change	p	E/N
Normal	2.10 ± .04	-	-	5.44 ± .10	-	-	2.59
Diabetic (Insulin)	2.76 ± .24	31	<.05	5.40 ± .31	(-7)	>.10	1.96
Diabetic (Diet Control)	2.08 ± .15	(-1)	>.10	4.14 ± .38	(-29)	<.05	1.99

3. On day 2 the net flux of the diet-controlled donor cells falls to 2.08 (a drop of 38%), and as a result there is no longer any significant difference between this value and the net flux of the normal cells, 4. The net flux of the diabetic subjects on insulin falls to a value of 2.76 on day 2 (a drop of 14%). However the net flux of these cells is still sitnificantly greater (31%) than the net flux of the control cells, 5. On day 2, the exchange flux of the diet-controlled diabetic cells exhibits a decline of 58% from its day-1 level and it is now significantly different from the exchange flux of the normal cells which only declines 13% over the 24-hour period, 6. The exchange flux of diabetic subjects on insulin declines some 32% over a 24-hour period. However, the day-2 flux of 5.40 is not significantly different from the day 2 flux of the normal cells.

In summary, the net fluxes of the cells of diabetic subjects on insulin remain at a substantially elevated level with respect to normal cells, while their respective exchange fluxes behave quite similarly to those of normal cells. The cells of diet-controlled diabetic subjects initially possess net flux values as high as those obtained from insulin-treated normal cells and exchange flux values in the normal range. However, both the net and exchange fluxes show dramatic reductions when assayed on cells after 24 hours' storage.

DISCUSSION

The control values which were obtained affirm both the methodology and the initial results which were previously reported (Mawe and Hempling, '65). The values for the net flux are quite stable with cells which have been stored for short periods of time. The exchange flux values show a somewhat lesser stability. Overall, the data indicate the presence of an average range of values to which experimental values may be compared as well as the necessity to have control values for each donor for comparison with experimental data as done in these experiments.

The flux data obtained with insulin-treated cells show that in general insulin elevates the net flux and has no significant effect on the exchange flux of glucose in human erythrocytes. Previous reports (Wilbrandt, '54 and Pletscher et al., '55) of net influx measurements in human erythrocytes indicated that insulin had no effect on red cell permeability to glucose. However, under the experimental conditions of the previous investigations, a maximal unidirectional net flux was not measured because during influx experiments the internal concentration of glucose in the cells rapidly exceeds the Km for glucose transport (Wilbrandt, '50). In the present experiments this difficulty was specifically eliminated by measuring the maximal net efflux into a medium which never approached the Km for glucose transport, thus providing more sensitive conditions for detection of an insulin effect.

Rieser and Rieser ('64) reported that insulin stimulates aldose-hexose transport into the human erythrocyte. Unlike the experiments reported here, this effect was obtained in the Rieser and Rieser ('64) experiments only after the red cells had been exposed to chymotrypsin for one hour at 38°C. They suggested that the proteolytic enzyme removed some substance

from the cell surface, allowing the red cell to become responsive to insulin with respect to the transport of aldose-hexoses. In the experiments reported here, a response of the maximal net flux to insulin without any other prior treatment of the cell has been observed. The levels of insulin utilized both in their experiments and those reported here are comparable when expressed as units per ml. cell water (Harris, '64). On such a basis, the insulin level of 0.9 units per ml. cell suspension (hematocrit 12.5 to 15%) used by Rieser and Rieser ('64) would be equivalent to 8.6 to 10.2 units per ml. cell water. The insulin levels used here, when similarly expressed, would range from 0.1 to 152 units per ml. cell water.

However, there are several differences in experimental techniques which may be responsible for the difference in observations reported in these two investigations: 1. In the experiment reported here the period of incubation with insulin is 30 minutes at 23°C. Rieser and Rieser ('64) incubated the cells for 15 minutes at 38°C., 2. In the experiments reported here red cells were preloaded with glucose prior to insulin treatment. In the efflux experiments reported by Rieser and Rieser ('64) cells were treated with chymotrypsin or chymotrypsin plus insulin before incubation with glucose, 3. Strikingly different methods for the analysis of glucose movement were used in the two investigations. The methods utilized in the present experiments, involving the rapid equilibrium distribution of an isotope of glucose and the calculation of fluxes from these initial glucose movements, may present a system more sensitive to discrete changes in the carrier mechanism.

Although several workers (Mawe, '56; Miller, '64 and Mawe and Hempling, '65) have shown that differences up to twice the tonicity between cell and external media (differences not encountered in the experiments reported

here) have no effect on the glucose flux in human erythrocytes, speculation may arise that the effect of insulin on maximal net efflux is related to the difference in tonicity between the glucose-equilibrated cell (395 mOsm per liter) and the glucose-free media (310 mOsm per liter). In several experiments the tonicity of the external media was adjusted with sodium chloride prior to measurement of the maximal net flux of untreated and insulin-treated cells. Under experimental conditions which were now essentially isosmotic, insulin still elevated the maximal net efflux to the same degree as noted previously.

The failure of heating to block the insulin effect completely does not seriously indicate a contaminant as the agent responsible for the elevation of the glucose flux. The usual elevation of the flux by insulin treatment is reduced by 80% by heating and it is quite possible that several moieties on the insulin molecule may be responsible for the flux enhancement and that some of these are not necessarily heat-denaturable. The fact that it is possible to reduce the insulin level five hundred-fold and still obtain almost the same degree of flux elevation suggests that the primary effective agent is the insulin and not any contaminant.

The question arises as to whether the effective concentration of insulin utilized in these experiments is comparable to that which is utilized in those experiments which treat with muscle preparations. For example, in frog sartorius muscle (Wohltmann et al., '67) the maximum permeability effect with 3-methyl glucose is obtained with 0.67×10^{-4} μmoles insulin per gram of muscle. By comparison, in the work reported here the threshold response begins between 3 and 30×10^{-4} umoles insulin per ml. packed cells. These insulin levels are best compared on the basis of molecules insulin per $\mathring{A}^2$ of surface area. The surface area of a single red cell is

145×10^8 Å^2 (Westerman et al., '61) and the number of cells per ml. of packed red cells is of the order of 1×10^{10} (Guyton, '66). Therefore the surface area of 1 ml. packed red cells is 1.45×10^{20} Å^2. The maximum surface area (Bloom and Fawcett, '62) of the myofibrils in one gram of sartorius muscle is approximately 3×10^{19} Å^2 (see appendix for calculations). Thus the maximum permeability effect in frog sartorius muscle is obtained with 1 molecule of insulin per 10^6 Å^2 surface area. By comparison the threshold effect in the red cell is obtained with from 1 to 12 molecules of insulin per 10^6 Å^2 of surface area. This represents at least a difference of one order of magnitude between the red cell and muscle requirement for insulin. However, considering the marked differences in the tissues, this difference in sensitivity to insulin is not unusually large.

Depending on the assay method and the ingestion of glucose, the plasma insulin levels in man cover a wide range (Guyton, '66). Fasting levels of 10-4000 µUnits per ml. plasma have been reported while assays made 1 - 2.5 hours after glucose ingestion yield insulin levels of from 50-20,000 µUnits per ml. plasma. On a basis of a specific activity of 25 units per mg. and an average hematocrit of 45%, a bio-assay value of 5000 µUnits per ml. plasma would correspond to 0.8×10^{-4} µmoles per ml. packed cells. As such, the amount of insulin added to packed cells in the experiments reported here is not enormously removed from the approximate apparent level of circulating plasma insulin.

The elevation of the maximal net efflux in contrast to the absence of an effect on the exchange flux cannot be accounted for on the basis of increased insulin binding inasmuch as the number of molecules of insulin bound to the red cell (Table 18) is the same both in the presence or

absence of glucose in the external medium. To effect an elevation of the net flux and at the same time not influence the exchange flux implies an effect directly on the free carrier. An effect on the binding capacity of the carrier for glucose would be evidenced by an alteration in the exchange flux. The same can be said for an effect on the movement or capacity of loaded carrier. Since the exchange flux is unaffected and the overall pattern of insulin is to raise the net flux value towards the level of the exchange flux, only the possibility that insulin interacts with free carrier appears capable of explaining the elevation of the maximal net flux.

The movement of the carrier-hexose complex across the cell membrane is considered to be the rate-limiting step. The difference in rates between the exchange and net fluxes obtained with normal non-treated cells has been attributed to the different mobilities of the complexed carrier and the free carrier. It has been suggested (Mawe and Hempling, '65) that free carrier moves some four times slower than complexed carrier. It is suggested here that the insulin elevation of the net flux is due on the whole, to an increase in the mobility of the free carrier. If the flux values obtained with insulin are inserted into a series of expressions utilized by Britton ('64) to demonstrate differences in carrier mobility, it can be shown that an insulin-elevated net flux is equivalent to a two-fold increase in the mobility of the free carrier. On the basis of the pore size values reported from water flux measurements it would be impossible for even a portion of the insulin molecule to enter the membrane (see appendix for calculations relating to the size of insulin). Solomon et al. ('68) have suggested the normal presence of water-filled pores of some 8-10 Å in diameter. Seeman ('67) has deomonstrated, with the use of ferritin, the

presence of transient pores of between 200 and 500 Å in diameter. The values obtained with ferritin, which were measured under conditions of slow hypotonic lysis, would allow a portion of the insulin chain to enter the membrane (assuming a linear configuration for insulin). However since the experimental conditions under which the fluxes are measured do not resemble those of hypotonic lysis (Seeman, '67) and inasmuch as insulin in solution does not exist in a single linear configuration but most likely as an aggregate of 2-4 units (Tietze and Neurath, '52 and Krahl, '60) the likelihood of insulin entering the red cell membrane is very small and thus the insulin effect would be expected to be a surface phenomenon. It is possible that when the carrier releases glucose at the outer surface under the experimental conditions designed to measure a maximal net flux, the free carrier takes on a different conformation. Such an alteration could result from being bound by sulphydryl groups within the membrane, bound by some other component, or influenced by charged groups (or lack of charged groups) on the surface or within the membrane. If such an alteration did occur the mobility of available carrier might be reduced without altering the concentration of free carrier or the amount of available carrier might be reduced through binding without affecting the mobility of the unbound carrier.

The observed effect of insulin may be to prevent such an alteration and the resultant apparent decline in mobility by acting either as a source to inhibit SH-disulphide interactions by entering into these reactions and protecting the free carrier, or as a polyelectrolyte offering either a charged surface for the free carrier to orient with or as a charged surface which can affect the membrane surface.

The number of molecules of insulin which bind to the red cell were determined under conditions resembling those under which maximal net and exchange flux measurements have been made (Table 18).

If the surface area covered by one insulin molecule in the extended configuration is approximated to be 2500 Å (appendix I), and this value is combined with the data from Table 18, it becomes possible to estimate what percentage of the cell surface could be occupied by insulin at the different initial concentrations. These estimations assume that insulin will be in an extended configuration, will exist in a non-aggregated state and will not bind solely by either the N-terminal or carboxy-terminal amino acids. These approximations attempt to recognize the maximum cell surface which could be covered with the quantity of insulin utilized in those experiments. It is recognized that the actual surface interacting with insulin could be a fraction of that which is indicated from the values which will be estimated. At an initial concentration of 3.1×10^{-3} µmoles per ml. packed cells, an average of 15 molecules of insulin bind to 1×10^{8} $Å^2$ of surface area. For an overall cell surface area of 145×10^{8} $Å^2$, this corresponds to 2200 molecules of insulin per cell occupying 6×10^{6} $Å^2$ of the cell surface. This last value represents 0.04% of the total cell surface. With this amount of bound insulin, a 33% elevation (Table 4) of the net flux is obtained. When the initial insulin level is raised a hundred-fold to 0.27 µmoles per ml. packed cells, the net flux is increased by 39%. At this concentration, 336×10^{3} molecules of insulin are bound per cell and approximately 5-10% of the cell surface may be covered with insulin. It is of interest that while the initial insulin concentration is increased a hundred-fold and the amount of insulin bound to the red cell is increased by 160 times, the net flux is only slightly increased

over the elevated value achieved with the lowest insulin concentration. The threshold of the response of the maximal net flux to insulin occurs somewhere between 0.3 and 3.0 x 10^{-3} µmoles insulin per ml. packed cells. This corresponds to the presence of 200 to 2000 molecules per cell occupying from 0.004 to 0.04% of the total cell surface. If it is assumed that the insulin molecule is reacting primarily with sites concerning glucose transport and that an increase of 40% in the net flux concerns essentially 40% of the sites, then the 40% elevation of the maximal net flux obtained with 3 x 10^{-3} µmoles of the insulin per ml. packed cells, indicates that the total number of sites occupy less than 0.1% of the total surface area. This value is approximately ten-fold lower than that estimated by Widdas ('54).

PCMBS

The area which a molecule of PCMBS can occupy may be approximated from molecular models (Fig. 14). The presence of the phenyl group makes PCMBS a relatively rigid molecule. If the primary locus of binding is between the mercury atom and a membrane sulphydryl, there are three possible conformations which the PCMBS molecule could assume. In one case, PCMBS would be perpendicular to the plane of the membrane, the mercury atom in contact with the membrane and the sulfonic acid group directed away from the membrane. In this case the minimum area of rotation which the PCMBS molecule would occupy would be approximately 20Å^2 and the minimum area taken by the mercury atom would be 8-10Å^2 at the site. A second conformation could occur if the molecule were oriented parallel to the membrane such that the plane of the benzene ring was perpendicular to the plane of the membrane. In this position the Hg atom and the sulfonic acid group could form a two point attachment with the membrane, though sterically, the formation of a bond between Hg and a membrane sulphydryl is not particularly favored in this position. The minimum area the PCMBS molecule would now occupy at

the points of attachment of Hg and SO_3OH would be approximately $20\AA^2$. A third possible conformation would occur if the plane of the molecule were parallel with the plane of the membrane surface and the mercury atom, the benzene ring and the sulfonic acid group then formed three sites of attachment possibly by means of covalent, hydrophobic and ionic attractions. The area occupied by the molecule in this case would be approximately 60-65 $\AA^2$.

Unlike PCMB which has also been shown to inhibit glucose transport in red cells, PCMBS does not penetrate the red cell membrane (van Steveninck et al., '65). Since both molecules occupy essentially the same molecular area, and an area of 20-30 $\AA^2$ would be sufficient for penetration it is most likely that this lack of penetration may be attributed to the substitution of the sulfonic acid group in PCMBS for the carboxyl group in PCMB and the resultant increases in the hydrophilic character of the former (Velick, '53).

At a PCMBS concentration of 0.05 μmoles per ml. packed cells (Table 7), the lowest concentration of PCMBS tested, the exchange flux is inhibited while the net flux is unaffected. At this level of PCMBS and depending on the particular orientation the PCMBS molecule assumes, a maximum of 0.4 - 1.3% of the surface area of the red cell could be covered by PCMBS. At a concentration of 0.7 μmoles PCMBS per ml. packed red cells, the exchange flux is brought to the level of the normal net flux, while at the same time, inhibition of the net flux first appears. At this PCMBS concentration, anywhere from 6 to 18% of the cell surface is covered. The maximum inhibitions of the exchange and net fluxes which can be obtained occur at PCMBS levels which correspond to a surface coverage of 33 to 97%. The value of $145 \times 10^8 \AA^2$ for the surface area of the human red cell (Westerman et al., '61) was used for these calculations. Interestingly,

the net flux, even in a cell which may be completely covered with the inhibitor, is never fully inhibited.

From Table 16, it can be seen that the number of molecules of PCMBS binding to the red cells is approximately in a linear relationship with the number of molecules added initially. When the data is combined with that obtained from the flux measurements, it becomes possible to approximate the number of sites on the membrane which are concerned with glucose transport. At the point where the net flux is unaffected while the exchange flux is brought to the level of the normal net flux, as few as 1 to 2 molecules per 10^4 $Å^2$ of the cell surface need be bound. If each molecule of PCMBS is bound to a single site, each of which is concerned with glucose flux, a maximum of $1.5 - 3.0 \times 10^6$ molecules of PCMBS per cell would be required to lower the exchange flux to the level of the normal net flux. Inasmuch as it can be demonstrated that more PCMBS may be bound to the surface even after the exchange flux has been brought to the level of the normal net flux, it is possible that a figure of $1.5 - 3.0 \times 10^6$ probably represents the maximum number of sites actually involved with glucose transfer at the cell surface. This figure for an upper limit in the number of glucose transport sites at the outer surface is in general agreement with the value of $1 - 1.4 \times 10^6$ suggested by van Steveninck et al. ('65) as the upper limit in the number of glucose transport sites. Depending on the conformation assumed by PCMBS with the membrane sulphydryl groups, a maximum of from 0.4 to 1.2% of the total surface area of the red cell would contain these reactive sites. These figures are essentially in agreement with the value of 1%, for the surface area containing the reactive sites, reported by Widdas ('54).

The inhibition of the maximal net flux is first apparent when the PCMBS level is such that anywhere from 4 to 11% of the red cell surface will be

occupied with bound PCMBS. In order to demonstrate 40 to 50% inhibition of the net flux, it is necessary to use sufficient PCMBS to practically cover the entire red cell surface. This inhibition can be visualized as either a prevention of glucose from leaving the carrier or a binding to the carrier in such a manner as to either prevent or slow the carrier's return to the interior surface. Since at the lower levels of PCMBS there does not appear to be any interference with the movement of the free carrier, it might be reasonable to assume that even at the higher PCMBS levels, there is still no interaction with free carrier and that the inhibition of the net flux is a result of PCMBS (at high concentrations), preventing the release of glucose from the loaded carrier at the outer membrane surface. However this assumption would now require PCMBS to prevent both glucose uptake as well as glucose release from the carrier. As an alternative, it is possible that PCMBS ties up carrier as the glucose is released at the outer surface. As a result the effective carrier concentration is reduced. The initial effect is seen in the readily obtainable inhibition of the exchange flux and subsequentially, as the level of PCMBS is markedly increased, in the inhibition of the maximal net flux. However it would be expected that if the carrier concentration was being decreased it would be possible to completely inhibit the maximal net flux. Since the maximal net flux is only partially inhibited, the likelihood of a decrease in carrier concentration appears to be small.

CHLORMERODRIN

By comparison to PCMBS, chlormerodrin is a less rigid molecule (Fig. 14) and is also capable of slowly penetrating the red cell membrane. Chlormerodrin is composed of four distinct groups, namely - a urea, a methoxy, a propyl and a metallic residue, and a number of conformations between the

molecule and the membrane are possible. For example, there may be a one-point attachment, Hg to membrane-SH, with the rest of the molecule in either an open or a packed configuration. The molecular area of a one-site open configuration would be approximately 50 $Å^2$ while a one-site packed area would be approximately 25 $Å^2$. A two-point attachment (possibly involving the urea moiety along with the Hg atom) with the rest of the molecule in either a packed or an open configuration is possible. A two-site configuration would take up approximately 50 $Å^2$ while a two-site packed configuration would occupy approximately 35 $Å^2$.

From the data in Tables 8 and 17, it can be seen that at a chlormerodrin concentration of 0.55 μmoles per ml. packed cells the exchange flux is brought to the level of the normal net flux value. Thus, with two molecules of chlormerodrin bound per 1000 $Å^2$ of cell surface, it is possible to demonstrate a 57% inhibition of the exchange flux as well as a 60% inhibition of the net flux. This amount of chlormerodrin is equivalent to 30×10^6 molecules per cell. If all the chlormerodrin were bound to the surface (and depending on the configuration assumed by chlormerodrin) anywhere from 5 to 10% of the surface area would be involved.

Unlike the results obtained with PCMBS, the net flux (even at the lowest concentration of chlormerodrin) is considerably inhibited. With PCMBS, 30×10^6 molecules bound per cell are required for the demonstration of a 27% inhibition of the net flux while with chlormerodrin, an equivalent inhibition is observed with 6×10^6 molecules bound per cell. This ability to inhibit the net flux could be considered to be a result of the ability of chlormerodrin to penetrate the membrane.

At 4°C the penetration of the membrane by chlormerodrin is considerably decreased. However a considerable degree of inhibition of both fluxes is

still evident (Table 8). In order to compare the data obtained with chlormerodrin at 4°C and at 22°C, it is necessary to take into account the fact that at 22°C, a quantity of chlormerodrin passes through the membrane and that the concentration of this chemical at the surface of the cell membrane is now considerably reduced. Therefore as seen in Table 8, a corrected "external" concentration of chlormerodrin of 0.59 µmoles per ml. packed cells at 22°C results in a 75% inhibition of the maximal net flux and a 73% inhibition of the exchange flux. With similar concentrations of chlormerodrin at 4°C the inhibition of the maximal net flux is 49% and the inhibition of the exchange flux is 59%.

From these observations, along with the data obtained with PCMBS, it is possible to conclude that the sites of glucose transport are not restricted to the surface layer as suggested by van Steveninck et al. ('65). A differential inhibition as witnessed by E/N ratios (Table 7) which decline is obtained with increasing amounts of PCMBS. On the other hand, with chlormerodrin both fluxes are similarly inhibited as seen from E/N ratios (Table 8) which remain relatively constant with increasing amounts of inhibition. These data tend to support the concept of a mobile carrier and suggest the presence of glucose transport sites not only on the external surface of the membrane but within and on the inner surface of the membrane as well.

In the experiments in which insulin is used in combination with the mercurial inhibitors (Tables 9-11) sufficient inhibitor has been added to reduce the level of the exchange flux to that of the net flux control value and also to interfere with the maximal net flux. Under these circumstances glucose in the medium may be prevented from recombining with free carrier. In addition it is also possible that free carrier may be

bound to PCMBS and that as a result, the mobility of the free carrier may be lessened. Under these circumstances both free and loaded carrier should be found in the membrane and a situation similar to that present under net flux will occur. Insulin under these circumstances could act in any one of three ways. 1. Insulin could act to increase the mobility of any free carrier which is present, 2. Insulin could act to increase the availability of free carrier or 3. Insulin could interfere with the PCMBS-inhibition of glucose recombination with the empty carrier. From the data in Table 9 dealing with the exchange flux, it can be seen that insulin (in the amounts used) causes a recovery by 50% of the inhibition of the exchange flux of glucose by PCMBS. This recovery of the PCMBS-inhibition may be due to the ability of insulin either to increase the free carrier mobility or to partially relieve the PCMBS-inhibition of the recombination of glucose with the empty carrier.

Under the conditions of a maximal net flux measurement, the glucose recombination factor would play no role. Insulin, under these conditions, can serve to increase the mobility of the free carrier or make more free-carrier available. Where the cells were first exposed to PCMBS (Table 9), insulin did not significantly reduce the PCMBS inhibition of the maximal net flux. Where insulin was added prior to, or simultaneously with PCMBS, the inhibition of the maximal net flux was prevented. These results may be accounted for if PCMBS binds more readily to free carrier than to loaded carrier. If insulin is present, free carrier may possess a different conformation, or to put it another way, insulin may mask the presence of free carrier and prevent a direct interaction with PCMBS.

There is no apparent correlation between the overall binding of PCMBS and/or insulin to the whole cell and the effects these molecules have on

glucose transport. The amount of PCMBS which binds to the red cell was not affected by the presence or absence of either glucose (Table 16) or insulin (Table 19) while the amount of insulin which binds to the red cell was decreased when cells had been previously treated with PCMBS (Table 21). It is possible that the manner in which the different agents arrange themselves on the membrane surface and their subsequent interactions with each other rather than the absolute quantities used are more significant to understanding the flux values obtained when these various agents are used.

Insulin is added to the cells under exchange conditions, it binds in small quantities similar to that which binds when glucose is absent (Table 18) but has no measurable effect on the flux (no free carrier is available). Let us say the addition of a large quantity of PCMBS may prevent the recombination of glucose with carrier. Free carrier would now be available and insulin apparently is now able to operate on what could be considered a simulated net flux condition. That is, the free carrier now present can be stimulated by insulin to pass across the membrane more rapidly. The inhibition of glucose recombination remains in full effect and therefore, though glucose is present in equal concentrations on both sides of the membrane, an apparent net-flux-like condition is maintained. This would, however, result in an apparent uphill net transfer of glucose. It is possible then, that insulin, in this instance, acts to lessen the PCMBS inhibition of the carrier-glucose recombination and that as a result, the exchange flux is increased. It may be that an experiment with PCMBS and insulin utilizing C^{14}- labeled glucose in the cell and tritiated glucose in the environment would provide data which would allow for a better explanation of what occurs when the exchange flux is inhibited

by PCMBS. Whether PCMBS is added first or whether insulin and PCMBS are added simultaneously, the same overall picture is observed (Table 9).

The insulin-chlormerodrin combination experiments were carried out at both 4°C and at 22°C. At both temperatures the level of chlormerodrin utilized was that which inhibited the net flux by 50% and which reduced the exchange flux to the normal maximal net flux value. At 4°C (Table 10) where chlormerodrin is a non-penetrant, the degree of the inhibition of the maximal net flux was not altered by insulin. On the other hand, the degree of the inhibition of the exchange flux was reduced by at least 50%. At 22-24°C the degree of inhibition of the exchange flux caused by chlormerodrim was not altered by insulin. The inhibition of the maximal net flux by chlormerodrin is partially relieved by the addition of insulin. On the other hand, the pre-incubation of the cells with insulin does not prevent the inhibition of the max imal net flux by chlormerodrin. In the case where insulin is the second component to be added, it may be that there is insufficient chlormerodrin to block all the carrier molecules, and those that are still free are capable of being stimulated by insulin. These results suggest that as was the case for PCMBS, where chlormerodrin is a non-penetrant, the relief of the inhibition of the net flux may be dependent both on insulin and on the presence of glucose, and that the action of insulin is probably directed primarily towards free carrier. The importance of the presence of glucose is suggested, since the inhibition of the flux when glucose is absent (maximal net flux conditions) is not affected by insulin (except for the one instance mentioned above, which may be an anomoly) while the inhibition of the flux when glucose is present (exchange flux conditions) is relieved by at least 50% by insulin (Table 10). It is also possible, that under the conditions where an

exchange flux is measured, insulin may act to prevent chlormerodrin-carrier interaction and that such "liberated" carrier as would now become available could combine with environmental glucose. The final result would be evidenced by an exchange flux with values more nearly resembling those of the control. The results obtained at the higher temperature, where chlormerodrin is a permeant, suggest that the interaction or chlormerodrin with carrier in the membrane may, as was suggested earlier, be more extensive than merely an outer surface interaction since the inhibition of the exchange in cells which have been exposed to chlormerodrin at 22-24°C is neither prevented nor relieved by insulin. As was the case with PCMBS, the amount of chlormerodrin which binds to the red cell is not influenced by the presence of insulin (Table 20) or glucose (Table 17). At the lower temperature, the amount of chlormerodrin which binds is decreased. The amount of insulin which binds to the red cell is decreased by the presence of chlormerodrin (Table 22). However, here also, the results of the binding experiment with chlormerodrin do not appear to offer any explanation of either the effect or lack of effect that insulin has on chlormerodrin-treated cells.

In general, the results obtained with insulin, the mercurials alone, and the mercurials in combination with insulin, indicate that sulphydryl groups as reported by others (LeFevre, '48 and van Steveninck et al., '65) are involved with glucose transfer but that these groups are not necessarily restricted solely to the outer face of the membrane (van Steveninck et al., '65). Measurements on the inhibition by mercurials of both the maximal net and the exchange flux in identical cell populations had not previously been made. The results obtained from binding measurements and inhibition studies of net fluxes led to the conclusion that the sulphydryl groups

involved with glucose transport were restricted to the outer face of the membrane. However, the differential results obtained on maximal net and exchange fluxes with PCMBS and chlormerodrin reported here indicate a reasonable probability that some of the sulphydryl groups involved in glucose transport are located within the membrane.

Unlike the results obtained here for the red cell, it has been reported (Wohltman et al., '67) that ACTH, oxytocin, vasopressin and the separated chains of oxidized insulin and of insulin-S-sulfonate did not alter the permeability of frog muscles to 3-O-methyl-D-glucose. The concentration levels utilized in the experiments reported here are several hundredfold greater than the quantities utilized by Wohltman et al., ('67) and this may account for the difference. However there are reports that these compounds do affect glucose permeability in rat hemidiaphragm (Ottaway, '53), isolated rat heards (Fisher and Zachariah, '60), and adipose tissue (Mirsky and Perisutti, '62).

The relationship between sulphydryl inhibition and insulin action is still not clear. It has been suggested (Edelman et al., '63) and Fong et al., '62) that insulin interacts with the membrane by means of thiol-disulphide interchanges. Several reports (Carlin and Hechter, '62 and Mirsky and Perisutti, '62) have indicated that pretreatment of the tissue with NEM and the resultant binding of membrane-sulphydryl groups has had no effect on the subsequent binding of I^{131} insulin. These results suggest that thiol-disulphide interaction is not the primary mechanism in all cases of insulin-tissue interaction.

The elevation of the maximal net flux, in a manner similar to that of insulin, by vasopressin and GSSG, which contain disulphide bonds, while GSH and cysteine were without any effect (Table 5) lends support to the

concept of thiol-disulfide interactions as the mechanism for insulin action on the membrane. However, the disulfide groups may be only one of several interaction sites. That this is possible as far as glucose transport in human red cells is concerned is evident from the effect on the net flux obtained with insulin derivatives (Table 6). These molecules with the exception of the S-Sulfo-A-chain, contain neither disulfide bridges nor sulphydryl groups (Figs. 9 and 10). In addition, the net flux is elevated by ACTH (30%) and by polyglutamic acid, M.W. = 2300, (20%). The amounts (in μmoles) of the various agents which were utilized in the flux experiments were combined with approximations of the maximum area ($Å^2$) that each would cover in their extended configurations. The spread between the agents was less than 10-fold and differences between active (flux affecting) agents and nonaactive (flux indifferent) agents were negligible. This suggests that the differences obtained with the various agents rest with the nature of the individual molecule and not merely with its size or the concentration in which it is used. The requirement of the presence of specific amino acids in oxytocin and vasopressin in order to obtain significant effects on water movements and on Na^+ transport has been demonstrated (Elliot, '68). These results further suggest that a physiological effect on a membrane involves more than thiol-disulfide interchange.

When red cells are exposed to phospholipase C for short periods of time, inhibition of the exchange flux (Table 12) and lysis (Table 14) can be demonstrated. With the concentrations of Phl-C which were utilized there was no effect on the maximal net flux. When the cells were pretreated with insulin the degree of lysis by Phl-C was considerably reduced and the inhibition of the exchange flux was prevented. In addition

the data reveal that the ability of the net flux component to be enhanced by insulin is unaffected by the presence of phospholipase C. The primary mode of action of phospholipase C on the red cell membrane is the hydrolysis of phosphatides with the resultant release of the phosphoryl nitrogenous moiety (Lenard and Singer, '68). It is suggested here that as a result of this digestion, Phl-C affects the exchange flux in a manner somewhat similar to that of PCMBS, in that either the enzyme, the enzymatic reaction products or an altered membrane protein conformation inhibits the recombination of glucose with free carrier at the outer surface. Unlike the results obtained with PCMBS, the addition of insulin to PhL-C-treated cells does not decrease the inhibition of the exchange flux. It appears that the phospholipase may not affect the free carrier since insulin can raise the net flux in cells which have been treated with Phl-C (Table 13). The ability of insulin to prevent the inhibition of the exchange flux gives rise to speculation that the insulin blocks in some manner the ability of the phospholipase to approach its phosphatide substrates on the membrane surface. Robinson ('66) has suggested that insulin reacts with cis double bonds of fatty acids and it is conceivable that such an interaction could interfere with phospholipase C degradation of membrane phosphatides. The action of insulin with relation to phospholipase C again indicates the probability that insulin-membrane interactions are not restricted solely to thiol-disulfide reactions.

In the past there have been numerous attempts to demonstrate that the carrier moiety involved with glucose transport in red cells is either a lipid or a protein. A number of these dealing with lipids (LeFevre et al., '64; Mawdsley and Widdas, '67 and Hobson and Laris, '66) and proteins (Widdas, '54; Bobinski and Stein, '66, and Forsling et al., '68), have already been mentioned. There has been no clear-cut demonstration

for the predominance of either moiety as the carrier directly involved with glucose transport in erythrocytes. Lenard and Singer ('68) have shown that 70% of the red cell membrane phosphorous can be removed by phospholipase C treatment and that the conformation of the structural proteins are unaffected. Nevertheless the role of the phosphatide in transport should not be overlooked. As shown in Table 12 the exchange flux of glucose transport is inhibited when red cells are exposed to phospholipase C. Though there is no evidence nor does it appear likely that the carrier is wholly or even substantially lipid, the results of these and other experiments (Odesser and Mawe '67), show that the phospholipid content and orientation play a considerable role in the proper functioning of a glucose transport system.

Recently, a considerable amount of data gathered from transport studies in micro organisms has provided ample evidence for the predominant role of protein in transport phenomena of these organisms. Kabach and Stadtman ('66) have demonstrated a proline transport system firmly linked to membrane fractions of E. coli. The transport system, β-galactocide permease of E. coli, has been actively studied and a specific site has been identified with the use of NEM (Fox et al., '67). The protein of this system is inducible and there are about 10,000 such "M proteins" per bacterium. This protein has been partly purified (Kolber and Stein, '66). A sulfate-binding protein has been purified and crystalized (Pardee, '66). This protein has a molecular weight of 32,000 and binds one sulfate per protein molecule. Mutants lacking this protein are unable to carry sulfate into the cell. Pardee ('68) has suggested that this protein could stretch across the membrane (70-120 Å) forming a passageway for sulfate transport. The entrance of glucose in certain strains of E. coli has been shown to be

dependent on a phospho-enolpyruvate (PEP)-linked enzyme system (Simoni et al., '67). The results of these investigations have given increased impetus to the study of the role which proteins play in transport.

The results of the investigation in microorganisms show both the involvement and the capability of proteins to carry out transport functions. There does not appear to be any reason to assume that proteins could not carry out similar functions in other cells. The experimental data show that in the red cell protein is involved, but that in addition, the lipid components play a definite role in the overall structural organization of the transport system. From an overall point of view the work reported here is consistent with the view that there appears to be a "partnership" involvement of protein and lipids in the transport of non-electrolytes in red cells.

It is not possible at the present time to positively attribute the flux results obtained with cells from diabetic donors directly to insulin. One can propose that for the donors on insulin therapy, there is a continuous and possibly high level of circulating insulin, and that as a result, the red cells are able to bind and hold, even after _in vitro_ washings, a quantity of this insulin and that this accounts for the elevated net flux values. The elevation of the net flux in the cells obtained from the "diet-controlled" diabetic donors cannot be readily accounted for at the present time. The marked instability of both the net and exchange fluxes in the stored cells (day 2, Table 24) from this group of donors suggested the presence of an altered and unstable surface configuration. The inability of insulin added _in vitro_ to affect the net flux in the cells obtained from both groups of donors may reflect either the presence of a surface already saturated with insulin, or a surface which is altered and will not accept (or be affected) by bovine insulin added _in vitro_.

It was not possible to obtain any data from uncontrolled diabetic subjects and one can only speculate as to what the control fluxes and the *in vitro* responses to insulin would be with cells obtained from such a group of donors.

It is as yet premature to suggest any practical application as a result of the data obtained on diabetics. All that can be said is that it seems that by measuring the glucose efflux under maximal net conditions, it would be possible to recognize a group of individuals who were both diabetic and were receiving insulin. Whether such a system of flux analysis could be used to measure either increased output or lack of insulin, or refractoriness to insulin, cannot be determined at the present time.

CONCLUSION

To briefly summarize, the results of the above work are as follows:

1. The maximal net flux of glucose-C^{14} across the human red cell membrane is on the average 2.3 millimoles per liter cell water-sec. The exchange flux of glucose-C^{14} across the human red cell membrane is on the average 6.0 millimoles per liter cell water-sec.

2. The maximal net flux values decline by 10% over a 48-hour period while the exchange flux declines on the average 15-20% in cells stored up to 48 hours.

3. Insulin raised the maximal net flux (in approximately 80 experiments) on the average by 49%. Insulin had no effect on the exchange flux.

4. The maximal net flux was also raised by the A-chains and b-chains of insulin, by vasopressin, oxidized glutathione, ACTH and polyglutamic acid. The exchange flux was unaffected by these agents.

5. The exchange flux of glucose-C^{14} across the human red cell membrane is readily inhibited by PCMBS.

6. The maximal net flux of glucose-C^{14} across the human red cell membrane is inhibited by PCMBS only after the concentration of this non-permeant mercurial is increased by more than ten-fold.

7. Both the exchange and maximal net fluxes are inhibited to approximately the same degree (on a percentage basis) by the mercurial chlormerodrin at 24°C (a temperature at which chlormerodrin slowly passes across the red cell membrane). At 4°C (a temperature at which chlormerodrin is essentially a non-permeant), the maximal net and exchange fluxes are inhibited but to a lesser extent than that which occurs at 24°C with an equivalent concentration of chlormerodrin on the external surface of the cell.

8. The inhibition of the fluxes by chlormerodrin is lessened in some instances when the cells are exposed to insulin. a. At 24°C the inhibition of the exchange flux that is obtained with chlormerodrin is unaffected by insulin while the degree of the inhibition of the maximal net flux appears to be dependent on the order of addition of insulin and inhibitor; i.e., cells which are treated with insulin before exposure to chlormerodrin do not demonstrate a lessening of the chlormerodrin inhibition. b. At 4°C the inhibition of the maximal net flux by chlormerodrin is unchanged while the inhibition of the exchange flux is reduced by 50-60% by insulin.

9. The degree of the inhibition of the exchange flux caused by PCMBS is reduced by 50% by insulin while the degree of the inhibition of the maximal net flux appears to be dependent on the order of addition of insulin and inhibitor; i.e., cells which are treated with insulin after exposure to PCMBS do not demonstrate a lessening of the PCMBS inhibition.

10. The binding of PCMBS to the red cell was measured. At the concentration at which the exchange flux is reduced to the level of the normal maximal net flux, (and where the maximal net flux is unaffected), approximately 0.5% of the total red cell surface is involved. The inhibition of the maximal net flux appears when approximately 10% of the cell surface is occupied by PCMBS.

11. The binding of chlormerodrin to the red cell was measured. At a chlormerodrin concentration where from 2.5 to 10% of the cell surface is occupied by this mercurial, the exchange flux is inhibited by 57% (brought to the level of a normal maximal net flux) and the maximal net flux is inhibited by 60%.

12. The binding of added insulin was measured with the use of porcine insulin-I^{131}. The amount of insulin which bound to the cell

is the same under exchange flux or maximal net flux conditions. The level of insulin with which a 40% elevation of the maximal net flux can be obtained corresponds to the coverage of 0.04% of the total surface area of the red cell.

13. Although insulin affects the degree of inhibition caused by the mercurials, insulin (regardless of the order of addition) has no effect on the number of molecules of either PCMBS or chlormerodrin which bind to the red cell. The amount of insulin, however, which binds to the red cell is reduced when the cells are first exposed to PCMBS. The amount of insulin which binds is reduced by chlormerodrin regardless of the order of exposure.

14. The action of the insulin and other activators of the maximal net flux, as well as that of PCMBS, occurs at the cell surface. Chlormerodrin may interact with glucose carrier moieties at both the outer and inner cell surfaces.

15. The mercurials may inhibit the exchange flux by preventing the recombination of medium glucose with free carrier or by decreasing the mobility of loaded carrier. The inhibition of the net flux (resulting from either a reduction in mobility of free carrier or a reduction of the concentration of carrier) may result from an interaction of the mercurial with the membrane or with the carrier.

16. The exchange flux is inhibited by phospholipase C. The maximal net flux is not affected. The inhibition of the exchange flux by phospholipase C is prevented when the cells are first exposed to insulin. The maximal net flux is elevated by 10-25% by insulin even in the presence of phospholipase C. The degree of hemolysis which occurs with the addition of phospholipase C is diminished when the red cells are first exposed to insulin.

17. The red cells obtained from human diabetic donors have markedly elevated maximal net flux values. The behavior of these cells in terms of storage and the addition of insulin *in vitro* is described.

18. The action of insulin, the insulin derivatives, and the other agents which elevated the maximal net flux indicates a role for molecular moieties in addition to thiols and disulfides.

19. It is suggested that insulin acts on the free carrier and that this action is directed to either increase the mobility of free carrier or increase the concentration of carrier. It may be that the conformation of the carrier molecule is dependent on the presence of glucose, such that the absence of glucose on one side of the membrane results in an alteration in the carrier which is evidenced by a lowered flux (maximal net flux), or conversely that the presence of glucose on both sides of the membrane maintains a carrier conformation which results in an accelerated glucose flux (exchange flux). Further the action of insulin, and the other agents which elevate the maximal net flux, may be explained by their ability to prevent the alteration in the carrier which occurs when glucose is not present on one side of the membrane.

20. Further support is given for the involvement of a mobile carrier in the facilitated transport of hexose in human red cells. As a result of the data obtained with the various agents (in particular, insulin, PCMBS, chlormerodrin and phospholipase C) employed in the experiments reported here, it is speculated that the carrier is essentially protein in nature and that lipids, and in particular phospholipids, are required to maintain a structural organization which allows for the proper functioning of the carrier-mediated transport of glucose in human red cells.

The work which has been reported here may prove to be of value in the attempt to identify, isolate and purify the entity responsible for the mediation of sugar transfer in red cells. These efforts will entail membrane digestion and fractionation and it is possible that the two different flux measurements as well as the action of the various agents on these fluxes will be of use in the attempt to identify and isolate the carrier moiety. An approach towards this end may be possible' even prior to the use of cell disruption methods. Drawing on the examples of the use of bacterial mutants, it is possible that the measurement of the maximal net and exchange fluxes on cells obtained from individuals with genetic abnormalities of the red cell (i.e. sperocytosis, hereditary congenital non-spherocytotic anemia, thalassemias) may provide cell membranes which behave differently with respect to glucose transport. The chances of a successful isolation of an entity involved with glucose transport may then be increased.

In addition, the results obtained with cells drawn from diabetic individuals indicate the possibility that the methods described above for the flux measurements may enable one to obtain more information on the state of insulin _in vivo_. For example, it is possible that where it has been suggested that the diabetic manifestation is due to the unavailability of insulin rather than to non-production of insulin, the red cell may play a role in the binding of insulin.

APPENDIX

1. Flux measurements: controls - statistical analysis of selected pairs.

The data obtained from normal donors were examined for significant differences by means of the "t" test. Comparisons were made on the basis of donor and *in vitro* storage time.

There is no significant difference among the average net flux values; however, the average exchange flux values obtained from cells which have stood for different times show a different picture. There is a significant difference in the exchange flux values of day-1 (6.25) and day-2 (5.44) cells ($p < .05$) and day-1 (6.25) and day-3 (5.25) cells ($p < .05$). There is no significant difference between the values from day-2 (5.44) and day-3 (5.25) cells ($p < .05$).

An analysis was made for significant differences in the net flux of cells from the same *in vitro* time period but from different donors. On day 1 there are no significant differences between donors. On day 2 there are significant differences between donor 5 and donors 1,2,3, and 7 ($p < .05$). On day 3 there are significant differences in the net flux values between donors 1 and 2 and donors 1 and 6 ($p < .01$).

A similar analysis by donors was made for the exchange flux values. The variation between donors at the various times are as follows: 1. For cells used on day 1, there are significant differences in the exchange fluxes between donors 1 and 2 and donors 2 and 4 ($p < .05$). 2. On day 2 there were no significant differences ($p < .05$) between the donors.
3. On day 3, a significant difference ($p < .05$) in the exchange flux exists between donors 1 and 5.

The variation of net flux values obtained from the cells of the same donor with respect to storage time was examined. The values obtained with

the cells of donor 1 are significantly different ($p < .01$) between days 1 and 2, and days 1 and 3. There was no significant difference in the net flux values of this donor's cells between day 2 and day 3. The values obtained with the cells of donor 2 are significantly different ($p < .01$) between day 1 and 2, and day 2 and 3. There was no significant difference in the values of the net flux between different days with any of the other donors.

The variation of exchange flux values obtained from the cells of the same donor with respect to storage time was also examined and here it becomes evident that the exchange flux component appears to be far more sensitive to standing (storage) than the net flux. Between the values obtained on day 1 and those of day 2, there were significant differences with the red cells of donor 1 ($p < .01$) and donor 2 ($p < .01$). Between day 1 and day 3 there are significant differences in the values obtained with the red cells of donor 1 ($p < .01$), and donor 5 ($p < .01$). The latter is the only donor whose cells give exchange flux values which are significantly different ($p < .01$) between day 2 and day 3. The overall picture is one of a large decline of the exchange flux in the first 24 hours of standing followed by a general leveling.

Examination of the E/N values indicates that for day 1 the ratio ranges from a low of 3.21 to a high of 3.91 and had an average value of 3.34. The day-2 E/N values have an equally narrow spread with a low of 3.05 and a high of 3.85. The average E/N for day 2 is 3.28. The day-3 E/N ratios range between a low of 2.36 and a high of 3.32 and have an average value of 3.13. The unusually low E/N of 2.36 reflects the marked increase in the exchange flux of donor 5.

Overall, the values obtained from non-treated red cells appear to show what could be described as normal donor variation; they do not show any

significant differences in the overall net flux values. There are decreases with time in the exchange flux values and it is difficult to attribute this decline to any factor in the methodology.

2. Calculation for the surface area of the myofibrils of 1 gm. of frog sartorius muscle.

A specific gravity of 1.0 is assumed and a volume of 1 cm^3 is assigned to 1 gm of the muscle. Sartorius muscle (Bloom and Fawcett, '62) consists of long fibers 10-100 microns in diameter. Each fiber may consist of 4-25 myofibrils, each 2-3 microns in diameter. In order to calculate a maximum surface area, it was assumed that the muscle was closely packed with fibers 10 microns in diameter and 1 cm (10^8Å) long. It was further assumed that each fiber consisted of 5 myofibrils each 2 microns in diameter and 1 cm long. Therefore, there are present 1×10^6 fibers or 5×10^6 myofibrils, in 1 gm of saetorius muscle. Each myofibril has a diameter of 2 microns or 2×10^4 Å and a length of 1 cm or 1×10^8Å. The formula for the surface area of a cylinder is:

$$(1) \quad A = 2 \pi r h + 2 \pi r^2$$

$$\text{or } A = (2)(3.14)(10^4)(10^8) + (2)(3.14)(10^4)^2 \ \text{Å}^2$$

$$= 6.28 \times 10^{12} + (6.28 \times 10^8)$$

$$= 6.28 \times 10^{12} \ \text{Å}^2 \text{ per cylinder}$$

$$\text{Total surface area} = 6.28 \times 10^{12} \times 5 \times 10^6$$

$$= 3.14 \times 10^{19} \ \text{Å}^2$$

3. Calculation of the size of insulin

Molecular models (Lapine) accurately scaled, 1Å = 1.5 cm, were utilized. The area of an amino acid pair without side chain contributions was calculated to be 50 Å^2. There are 25 such pairs giving an average of 1250 Å^2 area. The average side chain contribution was 25 Å^2 and there are 51

side chains resulting in a side-chain contribution of 1275 $Å^2$. As a result the surface area was taken as 2500 $Å^2$. This value was also obtained by determining (using the same models) the average area of each component amino acid and summing the values obtained. Assuming a linear configuration, a molecule of insulin (5800 M.W.) will occupy an area of 2500 $Å^2$.

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Herbert Zipper was born April 4, 1934 in Brooklyn, New York. Upon graduating from Stuyvesant H.S. in January 1951 he entered C. C. N. Y. where he majored in chemistry, receiving a Bachelor of Science degree in August 1954. He was employed as a research assistant in the Department of Microbiology at the College of Physicians and Surgeons, Columbia University, during which time he was in attendance at the Brooklyn College Graduate School in the Department of Chemistry. He received a Master of Arts degree in June 1957. Since 1957 he has been with the Brooklyn Veterans Administration Hospital and presently holds the position of research chemist in the Special Research Laboratory for Oral Tissue Metabolism.

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