CHARACTERISATION OF THE ~~ANALYSIS BY INHIBITOR STUDIES OF THE~~ KINETIC PARAMETERS OF FACILIATED ~~OF~~ HEXOSE TRANSFER IN THE HUMAN RED CELL BY INHIBITOR STUDIES

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ABSTRACT.

The transfer of glucose across the human erythrocyte membrane occurs by a process of facilitated diffusion, the mechanism of which is not yet fully understood.

In an attempt to provide information about the structure of the binding site, the apparent half-saturation constants of various glucose derivatives were determined by the glucose exit technique. The variation of these half-saturation constants with temperature showed that with the exception of cellobiose all the other glucose derivatives examined possessed the same ΔE (approximately 42 kJ mole^{-1}) whether they were transported by the carrier or not.

The examination of a previously untried glucose derivative, 4,6-O-ethylidene-α-D-glucopyranose showed that this substance penetrated the cell readily but did not do so on the glucose system although reacting strongly with it. Instead it was found to penetrate the membrane by a process of diffusion.

Using the properties of this substance it was found possible to inhibit glucose exchange independently from each side of the membrane. This disclosed a marked asymmetry of the system for its substrates, the half-saturation constant for glucose at the inner surface being ten times that for the outer surface at 16° C, whilst ethylidene glucose showed a forty-fold asymmetry under the same conditions.

Studies of the inhibition of sorbose transfer by various sugars showed that the half-saturation constants determined by this process were about 2½ times larger than those determined by the glucose exit procedure for transported sugars and 1½ times larger for non-transported sugars.

The action of Chlorpromazine on glucose exchange and sorbose transfer

was studied and it was found that whilst the former suffered negligible inhibition the latter was inhibited up to three times at the concentrations used.

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CHAPTER 1.

INTRODUCTION.

A cell is by definition an enclosed structure and relies for its existance on the presence of a containing envelope, without which its constituent materials would be free to disperse into the surrounding medium. In order to survive, however, the living cell requires a supply of energy-giving substances which could not be obtained if the envelope were completely impenetrable. The envelope must therefore be permeable to the passage of metabolic substances but at the same time must be able to prevent the loss of cell constituents. It was partly by the recognition of this phenomenon of semi-permeability that the existence of the plasma membrane was inferred. It soon became apparent, however, that the semi-permeability of the cell membrane was different from that of membranes prepared by chemical methods. Anomalous observations such as the findings of different distributions of similar substances between cells and media indicated that natural membranes had a greater selectivity than was shown by artificial preparations and attempts were made to explain this in terms of physical properties of the membrane but further studies showed that such a process could only be explained in terms of a biological system (Pfeffer, 1897. Chapter 4.)

The investigation of the plasma membrane, its structure and the manner in which it achieves selective permeability has been the subject of tremendous interest in recent years. In part this interest is due to a realization that changes in membrane permeability can control or alter the behaviour of cells and through them can effect the whole organism. However, the main cause of the rapid growth of research in this field is the improvement in the techniques available, which has taken place in the last 25 years. The development of thin-layer chromatography and gel

electrophoresis has enabled the components of the membrane to be separated and identified. Physicochemical methods such as nuclear magnetic resonance and infra-red spectroscopy (Chapman et al. 1971) have allowed the structure of these components to be studied while electron microscopy and X-ray analysis (Shipley, 1973) have indicated the way in which the components are arranged within the membrane. The relatively recent techniques by which artificial membranes and ionophores may be studied has permitted the investigation of model systems and the manner in which preparations of pure membrane components arrange themselves (see Eisenman 1972). With the increasing availability of radio-actively labelled material many of the problems involving the indentification of components have been resolved while much of the current information on the kinetics of membrane permeability has also been derived from work with isotopes. In all of these studies, the computer, with its capability for processing large amounts of data and calculating complex equations with extreme rapidity, has permitted the use of sophisticated techniques and has enabled various hypothetical systems to be examined.

Despite the advances in membranology that have occurred in recent years there are still areas of study where much more information is required to make a complete understanding possible. Of those special permeability systems which have been recognised, the hexose transfer mechanism in the human red cell is still not as well understood as might have been expected. It requires neither an external source of energy nor the presence of activating substances and would appear to be a relatively simple process but it may be this very simplicity that makes investigation difficult since the less complex the system is, the harder it will be to specifically identify its components. However, a full characterisation of the hexose transfer system in the human erythrocyte if it could be achieved, might provide a model upon which more complex transport processes could be based and would thus assist in the elucidation of permeability problems in other

tissues.

The Erythrocyte

The human erythrocyte is a biconcave disc 7 - 8 μm in diameter and 2.2 μm thick. It has a volume of about 90 μm^3 and a membrane thickness of 6 nm although Pinder (1972) has suggested that at the equator of the cell the thickness may be greater and this would explain why the cell takes a biconcave shape. Murphy (1965) has shown that the equatorial region of the membrane contains a higher concentration of cholesterol than the other regions and this would provide an alternative explanation of this phenomenon.

The erythrocyte is probably the most popular cell for the study of membrane properties and the reasons for this popularity are fairly obvious. The cells are easily obtained without recourse to surgical procedures and once obtained can be conveniently stored if necessary. They are isolated from other blood cells by simply washing with saline, the other cells forming a thin layer - the 'buffy coat' - on the surface of the packed red cells.

The mature erythrocyte is devoid of subcellular structures and this is important in kinetic studies where additional compartments will make calculations more complex, and in the preparation of pure membrane samples for analysis. Also it is possible to lyse the cells and to reseal them to produce ghosts with given interior contents and hence to study the inner surface of the membrane.

A further property which has been used extensively in this project is the ability of the red cell to change its volume in a linear manner with changes in osmotic concentration. This relationship holds only near isotonicity but as the cell is normally bi-concave this change in volume is achieved without a corresponding change in surface area of the cell.

One disadvantage with the erythrocyte is that the cells lack the ability to resynthesize proteins and are in consequence in a state of degeneration. This problem is more important where the accurate analysis of membrane components is intended than in the study of transport phenomenon though preventing the study of induced permeability changes such as studied in bacteria by Cohen & Monod (1957). It is possible that difficulties may arise if time-expired bank blood is used.

The Erythrocyte Membrane

Before we can form any ideas about the processes underlying transport phenomenon, it is necessary to understand the structure of the membrane across which the transport is taking place. At present there is little general agreement between investigators in this field but a picture of the membrane, its structure and composition is gradually developing.

Membrane Composition

The lipid nature of the cell membrane was first recognised by Overton at the end of the last century from the greater ability of lipid soluble substances to rapidly enter the cell, but it was not until recently that the exact composition of the membrane lipids became known. Analysis of plasma membranes by such techniques as thin-layer chromatography and infra-red spectroscopy have shown that there are some ten different types of lipid present and that their relative amounts are subject to considerable variation between membranes from different sources.

The most abundant lipid in the erythrocyte stroma is cholesterol (25% of total lipids) which accounts for nearly all the neutral lipid in this membrane. It is a rigid and relatively bulky molecule which if present in excess will reduce the fluidity of the membrane and decrease the rate of diffusion (Kroes & Ostwald, 1971).

The other major lipid components are phospholipids, the most common,

phosphatidylethanolamine, phosphatidylcholine (lecithin) and sphingomyelin each account for about 20% of the total lipid. Phosphatidylserine (11%) and traces (5% or less) of phosphatidylinositol, phasmalogens, cardiolipin and other phosphatides are also found (van Deenan & de Gier, 1964). The fatty acid moieties of these lipids are mainly of medium length (C_{18} - C_{20}) and about a third of them are unsaturated (Hanahan, 1969). It appears that they have a fairly rapid turnover rate since their composition in the membrane may be readily altered by dietary changes. These lipids with their long hydrophobic 'tails' are presumed to be the main structural material of the membrane (Singer & Nicolson,1972).

Although the membrane is thought of as a lipid structure, protein constitutes the larger proportion of its material, representing some 50% of the dry weight of the membrane compared with 42% for the lipid and 8% for the carbohydrate. The nature of the proteins in the erythrocyte stroma is still largely undetermined but the amino acid ratios have been worked out and do not appear to favour either hydrophyllic or hydrophobic types.

Of the proteins which have been identified the most abundant is spectrin. This is found attached to the inner surface of the membrane in the form of rods which resemble actin but which do not appear to have any ATPase activity (Marchesi et al, 1969). It is presumed that spectrin is a form of structural protein but this is not proven. Other proteins present include certain of the enzymes, for example glucose-6-phosphatase and Na^+ and K^+ activated ATPase but these are not as numerous as those found associated with mitochondrial membranes.

A third species of proteins which has not so far been identified in the red blood cell but which had been extracted from other types of membrane is the group of transport proteins. These appear to be rather small molecules with a molecular weight of the order of 3×10^4 but the way that they function has yet to be elucidated.

The carbohydrate units in the erythrocyte membrane appear to have an immunulogical role which explains their presence at the surface of the membrane. The free sugar is not found but various glycoproteins and glycolipids exist and have been characterised (Marchesi,1972; Sweeley et al, 1969). They consist of a chain of carbohydrate molecules in a specific order, which is anchored to the membrane bulk by the protein or lipid moiety.

The low molecular weight glycopeptide found on the surface of the red cell by Lote et al, (1972) might be a membrane component but since it can be extracted by washing with slightly hypertonic saline it seems more likely that it is adsorbed from the plasma.

Membrane Structure

Although the constituents of the membrane are fairly well recognised, the way in which they are arranged to form a semi-permeable barrier is far from clear. Despite differences in composition between membranes many investigators think that there is a basic structure common to most, if not all types of membrane and that the heterogeneity may be explained as variations on a basic structure.

The study of the arrangement of components within the membrane makes considerable demands on the techniques used in physical chemistry and much of the present information derives from studies involving such methods as circular dicroism, proton magnetic resonance and differential scanning calorimetry. All of these techniques have yielded important information about the way in which membrane components are structured and the manner in which they interact.

Following the early work of Langmuir (1917) on the structure of lipid films Gorter & Grendel (1925) extracted the lipid from erythrocytes and measured the area of film that it formed. They found that this was equal to about twice the area of the membrane from which the material had been extracted and therefore suggested that the membrane consisted of a lipid

bilayer - two lipid layers arranged with their hydrophobic ends in contact and their polar groups facing out into surrounding media.

This bilayer formed the basis of the well known model first put forward by Harvey & Danielli (1938) in which each surface of the lipid is covered with a layer of unfolded protein. The placement of protein was suggested by the findings that the membrane had a lower surface tension than would be expected for a lipid film and that the thickness of a bilayer was less than that of the membrane. This model was developed by Davson & Danielli (1943) and the sandwich structure proposed provides a ready explanation of the trilaminar appearance of stained membrane sections when seen under the electron microscope. From work performed on myelin Robertson (1964) developed this model as a basis for all types of biological membrane suggesting that the protein which covered the surface was in the β-configuration.

Although the lipid bilayer forms a useful basis for the further development of membrane structure the lack of special permeability properties of the simple form has led to the suggestion of different molecular arrangements. Sjöstrand (1963) observed a repeating structure in mitochondrial membranes from kidney tubules which had been stained with permanganate. These were interpreted (Lucy, 1964) as spherical lipid micelles, 40 Å in diameter, held in closely packed hexagonal array by hydrogen bonding. Some micelles might be replaced by enzymes while pores of 4 Å diameter would exist between the micelles. The development of other models involving sub-units such as that of Green & Perdue (1966) followed as more information about the membrane components was accumulated.

The demonstration by several groups of authors (e.g. Lenard & Singer, 1966) that at least half of the protein in the membrane was in the α-helix conformation led to the inclusion of globular proteins in membrane models instead of the flat sheets proposed by Davson & Danielli. One of the first

models to include such proteins was that of Wallach & Gordon (1968) in which the proteins were arranged in a square lattice with bilayer lipid cylinders. This type of model puts the lipid in contact with the surrounding media and explains why phospholipase and fluorescent lipid probes can react readily with the membrane. Zahler (1969) has suggested a similar but less rigid system in which globular proteins consisting of two polar regions joined by α-helices are held in a bilayer of lipid by interaction of the lipid with the hydrophobic surfaces of the α-helices.

A careful attempt to rationalise the available information has been made by Singer (1971) who has considered the thermodynamic properties of lipid-protein systems, postulating that the system with the lowest free energy will be the one most likely to exist in the membrane. From this postulate he has developed the lipid-globular protein mosaic model which differs from those of Zahler and of Wallach in that all the proteins are not assumed to penetrate the full thickness of the membrane.

In addition to the information derived from physicochemical studies, some interesting findings are due to the use of the relatively new technique of freeze-etching, by this means it is possible to fracture membranes along lines of weakness such as between interfaces. Shadowing the resultant surface with carbon or similar material provides a replica which can then be examined under the electron microscope.

Using this technique on the red cell Weinstein (1969) has revealed that the membrane is covered with large numbers of particles (membrane associated particles), the outer surface having more (380,000 per cell) than the inner (85,000 per cell). By labelling the surface of the membrane with ferritin Branton (1971) had shown that it is possible to freeze-cleave the membrane along an internal plane, apparently through the hydrophobic region and to show these particles penetrating to the centre of the membrane.

A reasonable assumption is that such particles represent the globular

proteins and this has been proved so by Engstrom (1970) who treated red cell ghosts with pronase and was able to remove virtually all of the particles but not all of the membrane proteins. The membrane associated particles probably account for about 50% of the proteins in the membrane. Removal of practically all the lipid from the membrane still leaves a trilaminar structure (Fleisher et. al., 1967) and thus it would appear that there are other proteins present in the lipid matrix.

It may be noted that freeze-etchings of the inner surface of freshly collected red cells show a network of fine filaments (Weinstein, 1969) which correspond to the loosely attached protein spectrin.

Facilitated Diffusion.

The term facilitated diffusion was first used by Danielli (1952) to describe those examples of biological transport which do not involve the utilisation of energy, for example the specialized transfer of glycerol in the erythrocyte, but which exhibit a strong similarity to the reactions of enzymes. This idea of an enzyme-like transport process led to the development of a set of criteria by which such a system could be identified. These criteria which have been listed by Stein (1967) are as follows:-

1). A substance which penetrates by a facilitated transfer system will move down its electrochemical gradient so as to achieve equilibrium.

2). The rate of penetration of the normal substrate will be greater, usually by several orders of magnitude, than would be expected from a consideration of its physicochemical properties.

3). The system will exhibit saturation kinetics provided a sufficiently high concentration of substrate is studied.

4). The system will show a greater or lesser degree of specificity amongst its substrates.

5). There will be competition between different substrates, the presence of one reducing the rate of transfer of the other.

6). The system may be inhibited by relatively low concentrations of certain substances but is not expected to be affected by metabolic poisons.

7). The rate of transfer may show a marked variation between tissues from different organs or different organisms.

8). In suitable conditions the presence of a second substrate may cause an acceleration of the flux of a first.

[In Chapter 4 we shall see the negation of these criteria used to prove that a substance does not penetrate the membrane by facilitated diffusion].

The correlation of the properties of the hexose transfer system with the above criteria has been adequately reviewed by several authors, see for example Widdas (1963).

There has been considerable discussion in the past as to the form which the facilitated diffusion mechanism takes and as will be seen below, this problem is at present very far from being solved. In 1933, Osterhout first introduced the concept of a 'carrier', a molecule which could complex with a substrate on one side of the membrane and then cross the membrane by some means, releasing the substrate at the other surface.

Although certain authors (e.g. Lieb & Stein, 1972) have rejected the term 'carrier' because early models of this type do not fit all of the present data, we shall continue to use it, intending the term to refer to any system in which the binding site of the membrane component changes its position or the direction it faces during the translocation of sugars.

Isolation of the Carrier.

The success of workers in extracting enzymes from cell membranes led to attempts to isolate the membrane components responsible for sugar transport in the erythrocyte. The earliest approach appears to be that of LeFevre, Habish, Hess & Hudson (1964) who found that phospholipids extracted from the red cell membrane were able to solubilise glucose in non-polar solvents such as benzene. This work was extended by LeFevre, Jung & Chaney (1968) who

studied the enhancement of glucose transfer across a chloroform layer by membrane extracts. Mawdsley & Widdas (1967) also extracted a lipid from the membrane which increased the lipid 'solubility' of glucose and was influenced by DNFB and Hg^{++}. However such systems did not generally show the specificity expected for the glucose carrier as between d- and l-glucose for example (LeFevre et al. 1968).

The alternative to the lipid carrier is an enzyme-type structure. Considerable success has been achieved in the isolation of the so-called permeases in bacterial membranes. These, however, are rather easier to study than the carrier since their presence may be induced in the membrane by suitable treatment.

Attempts by Bonsall & Hunt (1966) to extract the carrier protein and by Bobinski & Stein (1966) to demonstrate its binding ability by retardation chromatography gave positive results. LeFevre & Masiak (1970) showed, however, that such results were spurius since mechanical agitation of the chromatography column or similar treatment could influence the results. Following examination of the column these authors suggest that Bobinski & Stein's results were due to the presence of membrane vesicles in their preparation which trapped quantities of solute. Masiak & LeFevre (1972) also found that a further attempt by Levine & Stein (1967) to demonstrate the specific binding of D-glucose by membrane extracts could not be reproduced but they were unable to provide a reason for Stein's original observation.

The lack of specific binding of glucose by protein extracts in LeFevre's experiments was thought surprising since about 90% of the membrane protein has been recovered but this does not seem so unlikely in the light of recent experiments by Jung et al.,(1973) who treated ghosts with pronase to remove 96% of the protein and still found the hexose carrier system intact. Because of this these authors have suggested that the carrier may be phospholipid in structure.

Kahlenburg et al., (1971) have developed a method for the measurement of glucose uptake by the intact erythrocyte membrane. They have investigated the effect of certain phospholipases on their preparation and have found that phospholipase A_2 will reduce the uptake of glucose by 75% but that phospholipase C has no effect. This fact might by construed as further evidence for the carrier being a lipid structure but Chapman (1973) has reviewed a large volume of data which indicates that interference with membrane lipids will result in changes in the activity of various membrane associated enzymes. Thus the possibility of a protein carrier buried in a lipid matrix is still tenable.

Obviously the removal of the carrier from its normal environment will prevent the demonstration of its transport properties and unless the extracted material can be reactivated by placement in an artificial membrane system, the only method of indentifying the carrier components will be by specifically labelling them before extraction.

This has been attempted by Stein (1964) and by Eady & Widdas (1973) using fluorodinitrobenzene (FDNB). Neither attempt was successful, there being too much non-specific binding to permit isolation of the carrier components. However, the latter authors used the differential binding of the inhibitor to ghost membranes in the presence of transported and non-transported sugars and this seems a promising method if sufficient material can be obtained to allow further analysis.

Development of the Carrier Model by Kinetic Studies.

[In this section the calculations of various authors have been standardised using the following set of symbols:- C, the concentration of sugar in the outside medium; S, the amount of sugar inside the cell in isoquantities; I, the concentration of inhibitor; V, the volume of the cell relative to isotonic volume, V_o; K, the maximal rate of transfer of sugar; θ, the

fraction of carriers which are saturated; φ, the half-saturation constant for transfer (K_m); σ, the dissociation constant for the carrier-sugar complex (K_s) and K_i, the inhibition constant.]

Although earlier authors, e.g. Klinghoffer (1935) had shown that glucose penetration did not follow Fick's law, the first attempt to describe it in terms of carrier transport appears to be that of LeFevre (1948) who considered that the glucose complexed with a membrane component of limited concentration and thus its rate of entry did not depend just on its own concentration. Later LeFevre & LeFevre (1952) derived equations for such a system in which the breakdown of the complex at the inner surface of the membrane was the rate-limiting stage of the process. This gave an equation :-

$$\frac{ds}{dt} = K\left(\frac{C - S/V}{C}\right)$$

for the rate of transfer of glucose and was shown by the authors to give a good fit to the empirical data, provided C was less than about 0.8 isotonic units (240 mM). They also showed that the dissociation constant of glucose at the outside of the membrane must be small.

At about this same time Widdas (1952) was developing a somewhat similar type of model. This was also based on the assumption that there were a limited number of sites in the membrane on to which sugars were preferentially adsorped but in this case it was the translocation of the complexed carrier across the membrane which was rate-limiting. The rate of transport into the cell will thus be proportional to the number of sites that are occupied on the outer surface of the membrane whilst the efflux of sugar will bear the same relationship to the degree of saturation of the inner surface of the membrane. Therefore, the net rate of transfer $\frac{ds}{dt}$, will be given by :-

$$\frac{ds}{dt} = K(\theta_o - \theta_i),$$

which can be related to the respective concentrations by Langmuir's adsorption

isotherm, $\theta = C/(C+\varphi)$. Thus the net flux becomes :-

$$\frac{ds}{dt} = K\left(\frac{C}{C+\varphi} - \frac{S/V}{S/V+\varphi}\right)$$

(In the true adsorption isotherm the dissociation constant (σ) should be used instead of the half-saturation constant (φ), but it has been shown by Dawson & Widdas (1964) that the presence of a transfer term in the above equation makes the use of φ correct in this instance).

Now the net flux equation may be simplified in two ways :-

If $\varphi \gg C$ and S/V then

$$\frac{ds}{dt} = K\left(\frac{C}{\varphi} - \frac{S/V}{\varphi}\right) = \frac{K}{\varphi}(C-S/V).$$

This equation is the same as Fick's equation and offers an explanation as to why the ketose sugars such as sorbose and fructose appear to enter the red cell by diffusion but are inhibited in the presence of glucose.

If $\varphi \ll C$ and S/V then

$$\frac{ds}{dt} = K\left[\left(1 - \frac{\varphi}{C}\right) - \left(1 - \frac{\varphi}{S/V}\right)\right] = K\varphi\left(\frac{1}{S/V} - \frac{1}{C}\right).$$

This equation indicates that for the same concentration gradient the rate of transfer is faster at lower values of S/V and C and this explains why the apparent permeability coefficient of high affinity sugars decreases at high concentrations. It was found that the results for glucose entry were fitted very well by this equation over the whole range of concentrations studied (Widdas, 1954a) and this model therefore was preferred to that of LeFevre.

Since sorbose has a very low affinity for the carrier its inhibition by glucose provides a useful means of measuring the half-saturation constant of the latter sugar. The fraction of sites saturated in the presence of a competitive inhibitor is given by :-

$$\theta = \frac{C}{C + \varphi + \varphi I/K_i}.$$

Thus the equation for sorbose transfer becomes, in the presence of glucose:-

$$\frac{ds}{dt} = \frac{K}{\varphi + \varphi I/\varphi_i} (C - S/V)$$

and a plot of the reciprocal of the rate against I will give a straight line with an intercept on the absciasa equal to $-\varphi$. Using this method Widdas (1954) obtained a value for the half-saturation constant for glucose of 7 - 17 mM.

A modification of the Widdas model was put forward by Wilbrandt & Rosenberg (1951) who suggested that enzymes might be involved in converting the sugar to a transportable form. This idea was supported by evidence obtained by Wilbrandt (1954) indicating that polyphloretin phosphate caused a greater inhibition of glucose exit than it did of glucose entry. This was interpreted as the result of asymmetry of the system but Bowyer & Widdas (1958) showed that in part this effect could be explained in terms of errors introduced by approximations in the equation used to determine the fluxes.

An alternative to the oscillating carrier is the polar pore in which sugars move along a chain of sites fixed to the sides of the pore. Bowyer & Widdas (1958) have shown that this exhibits the same kinetics as the simple carrier and the enzyme system. A possible way of distinguishing between the polar pore and the mobile carriers follows from a prediction of Widdas (1952) that in certain circumstances a mobile system will allow the accumulation of a sugar against its concentration gradient. Uphill transfer by counterflow as it is called is brought about in the following manner, if there is an unequal distribution of substrate across the membrane, a carrier will transport the substrate down its concentration gradient from the side of high saturation to the side of low saturation. At the low saturation surface the carriers will lose the sugar molecules they have transferred and because of their mobility the carriers will return to their original positions, a proportion of them bearing substrate from the side of low saturation. On being released at the high saturated surface these substrate molecules will have to compete with the higher concentration of substrate already present

and ~~S6~~ 80 will not be transferred back. They will thus accumulate until their specific concentration equals that of the substrate already there, after which time both substrates will flow down their respective concentration gradients until equilibrium is reached. Because the exchange of sugar is more rapid than the net flow the degree of accumulation of the low concentration substrate can be considerable, rising to more than ten times the equilibrium concentration.

Although this occurs with an uneven distribution of one substrate, two identifiable substrates must be used if the effect is to be examined experimentally. The first test of the prediction was performed by Park et al., (1956) using rabbit red cells which have a very slow rate of transport. A non-metabolisable sugar (xylose) was allowed to equilibrate with the cells and a solution of glucose was then added to the suspension. Estimations of the amount of xylose in the cells showed that it had dropped below equilibrium value due to the entry of glucose into the cells. Using radio-active sugars Rosenberg & Wilbrandt (1957a) were able to demonstrate the same phenomenon in the human red cell and thus, at this time the evidence was very strongly in favour of the simple carrier model described by Widdas.

The first evidence of a discrepancy between this model and the experimental data arose from the developement of the glucose exit technique by Sen & Widdas (1962a). In this technique the cell is preloaded with glucose at a moderately high concentration and then resuspended in solutions of a lower concentration. The loss of sugar from the cell is linear until near equilibrium. If the linear part is assumed to represent the maximal rate of transport then in the presence of external sugar this rate is reduced by the production of an opposing influx. The concentration of external sugar which reduces the rate to half that in the absence of external sugar will represent the half-saturation concentration (φ).

Sen & Widdas studied the variation of this parameter with temperature and pH and gave it a value of 4 mM at 37°C and pH 7.4. This value is several times smaller than the value for the same parameter obtained by Widdas for inhibition of sorbose transfer but this discrepancy was assumed to arise from errors involved in the equations used to measure the traces.

A more serious discrepancy in the simple carrier model was presented independantly in 1965 by Mawe & Hempling and Levine, Oxender & Stein (1965). These groups of authors confirmed an observation by Britton (1964) that the rate of glucose equilibrium exchange was faster than the rate of net flow. This is contrary to the simple carrier theory which assumes that the carrier moves at constant rate. To overcome this, both groups of authors suggested that there was an increase in the translocation rate of the loaded carrier and derived equations to enable the calculation of the relative rates of loaded and empty carriers.

The equation for efflux is:-

$$\frac{ds}{dt} = \frac{KS(rC + \sigma)}{(\sigma+C)(\sigma+rS)+(\sigma+S)(\sigma+rC)}$$

and from this we have an equation for net flux

$$\frac{ds}{dt} = \frac{K\sigma(C - S)}{(\sigma+C)(\sigma+rS)+(\sigma+S)(\sigma+rC)}$$

For the exchange where S = C, the unidirectional flux will be given by

$$\frac{ds}{dt} = \frac{KC(rC+\sigma)}{2(\sigma+C)(\sigma+rC)} = \frac{KC}{2(\sigma+C)}$$

from which it will be seen that the maximum rate will equal K/2, whilst the Sen-Widdas procedure where S=∞will give a net flux

$$\frac{ds}{dt} = \frac{K\sigma}{2rC+r\sigma+\sigma}$$

from which the maximum rate will be K/r+1.

Thus the ratio of the maximum rate of exchange to that of exit will be (r+1)/2 from which the value of a r may be measured. The two groups of authors obtained slightly differing values for r of 2.8 and 4.0.

Inspection of the half-saturating concentrations for the two processes shows that for exchange this parameter will equal σ, (the dissociation constant) while for exit it takes the form $(r+1)\sigma/2r$. Thus if $r = 1$ the half-saturating constant for exits is the same as for exchange (i.e. simple model) but if $r \rightarrow \infty$ then this term becomes $\frac{1}{2}\sigma$.

This result is important since it is the basis for a further criterion of transfer by a diffusing carrier.

Levine & Stein (1966) measured the rates of exchange and exit for various concentrations of glucose. In addition to the difference in transfer rates for the two processes, these authors also noted a difference in the values of the half-saturation constants. According to the kinetics given above this parameter for exits may vary between 0.5 and 1.0 times that for exchange. Levine & Stein, however, obtained values of 3 mM and 11mM respectively for these parameters at 25°C, i.e. the ratio of the half-saturation constants for exits and exchange is outside the range predicted for the above model. This finding and the other observation of these authors that the temperature dependence of the half-saturation constant for exchange was the reverse of that determined by Sen & Widdas (1962a) for exit are very difficult to reconcile with the simple carrier model.

In a series of standardised experiments intended to reduce methodological errors, Miller (1968a,b) confirmed the respective observations of Sen & Widdas and of Levine & Stein that the half-saturation constant for glucose exit was less than that derived from sorbose inhibition and also less than that for glucose exchange. He showed that neither the original carrier model nor the fast-complex model would give an adequate description of the observed effects in a counter-transport experiment.

In the same papers Miller determined the rates of exchange of different pairs of sugars and found that when the same sugar was on each side of the

membrane the rate of exchange was less than when the sugar pairs were different

In order to provide an explanation of these new measurements particularly Miller's results with different sugars, Naftalin (1970) proposed a lattice pore model similar to the polar pore considered by Bowyer & Widdas (1958) but permitting sugars on adjacent sites to exchange. Computer analysis of this model gave reasonable account of the observed phenomena including the difference in temperature coefficients of the apparent half-saturation constants and the faster rates of exchange for two different sugar species.

A factor often considered when existing kinetics do not give a good explanation of membrane transfer is the effect of a static layer of solution in contact with the surface of the membrane. This would produce a different concentration in the vicinity of the membrane to that in the bulk. This was examined by Naftalin (1971) who found that vigorous stirring could accelerate transfer. Later, however, Miller (1972) and Lieb & Stein (1972) both showed on theoretical grounds that to produce an effect the unstirred layer would have to be about 300 times the volume of the cell suggesting that Naftalin's results were due to some other effect, perhaps inadequate dispersion of the cells at the start of the experiment.

A further modification of the simple carrier proposed by Geck (1971) was the removal of the symmetry constraint. This modification had been previously examined by Regen and Morgan (1964) but using rabbit erythrocytes they have found no evidence to support the idea. Geck proposed that the change in affinity of the carriers as they crossed the membrane was compensated by a change in the resistance of the carriers to translocation. Unfortunately, Geck derived his parameters from experiments performed by Lacko et al., (1972) in which the rate of net flux of sugar into the cell was measured. This is a questionable procedure since as soon as influx starts a reverse flow of sugar will occur, consequently the apparent rate of entry is much less than the actual rate. Despite the unsuitability of influx velocity determinations

Hankin et. al., (1972) have used them in an attempt to reject the asymmetric model in favour of the Lieb-Stein tetramer (see below) Hankin's results suggest a value for the initial entry rate which is about half that obtained for 'zero-trans' exits but the Lieb-Stein model is symmetric over all and to fit this, one would expect the rates to be the same in both directions. However, Lieb & Stein (1972) have shown in general terms that any form of the simple carrier is incompatible with the empirical data.

Another of the models proposed at this time was the tetrameric model of Lieb & Stein (1971a,b). This consists of two pairs of sites one pair on each surface of the membrane. One site of each pair has a high affinity for the substrate and the other has a low affinity. Each high affinity site is situated opposite one with low affinity with which it may react although in the original model (Lieb & Stein, 1970) they were separated by a central pool common to all four subunits. Transport takes place by the introversion and reversion of subunits the sugar passing from one site to another according to simple probability theory. The rate of introversion depends on the occupancy of the four sites.

This model gives a qualitative explanation of most empirical observations including those produced by the so-called zero-trans experiments (Karlish et. al., 1972; Miller, 1971). These are superficially similar to the Sen-Widdas experiments but the outside medium ('trans' solution) is maintained as close as possible to zero concentration and the rate of transfer of glucose from different internal concentrations is measured. This procedure would be expected to give the same values for the transport parameters as the Sen-Widdas method but although the maximal transfer rate is the same the half-saturation constant is 14 times greater at 20°C. The explanation of this according to the tetramer model is that both internal units of the tetramer are saturated but transfer is more likely to occur from the low

affinity site inside to the high affinity site at the outer surface, than from the high affinity site inside to the low affinity site outside, therefore since the external concentration is zero the parameters will relate to the low affinity site on the inside (Lieb & Stein, 1972).

Investigating the anomalous findings of Miller (1968a) regarding the exchange rates of different sugar species Eilam & Stein (1972) found that in part Miller's results were due to the use of the same concentration of each sugar irrespective of the half-saturation constants thus, the saturation of the carriers was different in each experiment which made the rates of exchange appear different. By using integrated equations for the exchange process, Eilam & Stein showed that'heteroexchange' was not anomalous but confirmed that galactose was transported more rapidly than mannose which in turn was transfered faster than glucose.

The most recent model put forward for hexose transfer is LeFevre's (1972) introversion model in which the surfaces of the membrane contain sites normally facing the media but capable of introverting in the presence of sugar. Transfer occurs between introverted sites and it is the occupancy of the site that affects the degree of introversion. Using a computer LeFevre has shown that this model will give similar kinetics to other successful models.

At the present time, therefore, there are several possible mechanisms for transport which agree with the main kinetic observations and some attempts must be made to obtain further information which will allow them to be distinguished.

Inhibitor Studies

The reaction of substances other than the normal substrate with the carrier system is an important means of examining the various steps in the transport process. The use of substrate analogues offers a method of determining the structure of the carrier binding site while the use of

compounds which react with other parts of the carrier may provide information about the mechanisms of transfer.

Inhibitors are normally described in terms of the reversibility of their action and whether or not they compete with the substrate for attachment to the carrier. A reversible inhibitor forms a dissociable complex with the carrier molecule and its attachment will be described by an equilibrium or inhibitor constant (K_i) which is equivalent to the dissociation constant (K_s) used for the substrate. Such an inhibitor will therefore only be able to block transport completely if present at an infinite concentration. An irreversible inhibitor, on the other hand, can associate with the carriers covalently and provided it is present in excess it would be expected to inhibit the system completely. Its reaction is expressed in terms of the rate at which the inhibition develops under given conditions.

Competitive inhibitors react with the carrier at the binding site and will be affected by the presence of substrate while non-competitive inhibitors will be unaffected since they react elsewhere on the carrier. These two types of inhibitor may therefore be distinguished by examining their effectiveness in the presence and absence of a suitable substrate.

An irreversible inhibitor will show an effect by a change in the inhibited rate constant but a reversible inhibitor will react with the available carriers according to the equation already used to determine substrate binding, i.e. the fraction of sites inhibited may be represented by

$$\theta_i = \frac{I}{I+K_i}$$

If the inhibitor is reversible but non-competitive then the substrate will only be able to react with the remaining fraction of sites. Thus sites bound to substrate,

$$\theta_s' = (1-\theta_i).\theta_s = \frac{C/\varphi}{(1+I/K)(1+C/\varphi)}$$

If the inhibitor is competitive, the substrate will be able to displace the inhibitor from some of the sites it occupies thus the saturation of the substrate will be given by,

$$\theta_s^1 = \frac{C/\varphi}{1+C/\varphi+I/K_i}$$

Thus, if a Lineweaver-Burk plot is drawn of 1/rate versus 1/C for each inhibitor concentration, I, a series of lines each with two intercepts will be obtained.

A. For a <u>non-competitive</u> inhibitor the intercept on the ordinate will increase by a factor of $(1+I/K_i)$ whilst that on the abscissa remains unaltered.

B. For a <u>competitive</u> inhibitor the intercept on the ordinate remains unchanged but that on the abscissa decreases by a factor of $1/(1+I/K_i)$.

In addition to these two types of inhibition a third intermediate type is recognised termed allosteric or mixed inhibition. In this it is presumed that although the inhibitor does not attack the substrate binding site its reaction with the carrier is decreased by the presence of substrate. This will cause a change in both intercepts of the above plot making the K_i for the inhibitor more difficult to determine since alteration of the half-saturation of the substrate will produce a corresponding change in the apparent K_i (see Dixon & Webb 1964).

Since glucose transfer is a facilitated process it is not affected by metabolic inhibitors like iodoacetate (Wilbrandt et al., 1947) but it is sensitive to general protein reagents particularly those which react with thiol-groups. These include mercuric ions and organic mercurials such as para-chloromercuribenzoate (LeFevre, 1948) gold chloride and certain lachrymators (Wilbrandt, 1950) but not Cu^{++}, Pb^{++} or arsenic compounds (LeFevre, ibid). Inhibition due to thiol-group reagents is non-competitive although the activity of the carrier may be restored by treatment with high

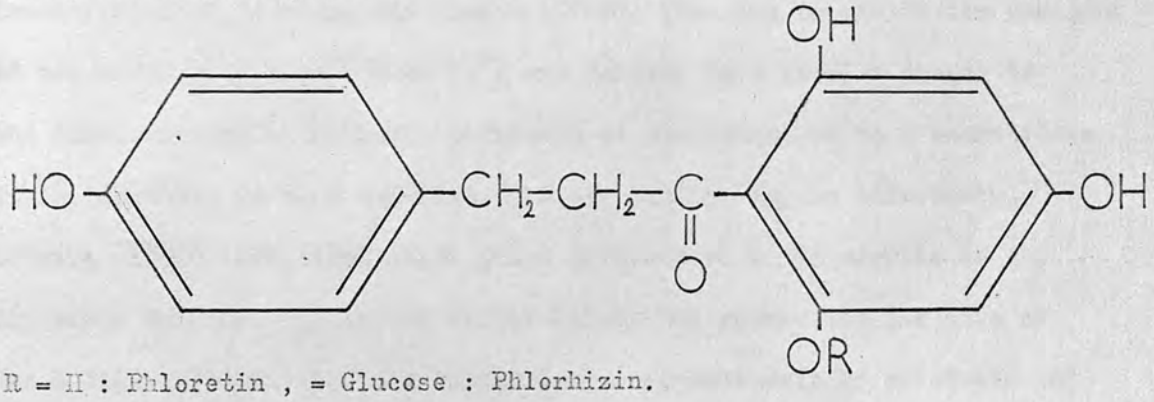
concentrations of SH-donating compounds such as cysteine (LeFevre 1948). 1-fluoro-2,4-dinitrobenzene (Bowyer 1954) is an irreversible protein reagent but this reacts with amino and phenolic hydroxyl groups as well as thiol groups and is unusual in that its inhibition developes at a faster rate if glucose is present (Bowyer & Widdas 1956). Krupka (1971a,b) has recently studied the reaction of FDNB in more detail and has shown that during the reaction the association of one molecule of inhibitor with the carrier assists the binding of a second molecule so that although Bowyer & Widdas (1956) found a complex reaction between carrier and inhibitor, a 1:1 stoichiometry probably exists. Krupka also showed that the facilitation of the binding reaction in the presence of substrate depends on the substrate being transported. Non-transportable substrates such as the disaccharides are able to protect the carrier against inhibition. This was interpreted as the result of a conformational change during transport which permitted binding of FDNB to occur. Krupka (1972) has shown by incubating cells with FDNB in the presence of a transported and a non-transported sugar that the resulting inhibition is compatible either with a carrier process in which uptake of the substrate can only occur at one surface at a time or with a highly asymmetric carrier which is sensitive to FDNB only at the outer surface. Edwards (1973), however, claims evidence to suggest that the inhibitor binds at the inner surface of the membrane.

Dawson & Widdas (1963) investigating the effect of N-ethyl maleimide (NEM) another SH-reagent with a similar action to FDNB, found that inhibition developed in two phases. Since NEM is very specific, the action being blocked by pretreatment with p-chloromercuribenzoate, this behaviour has been taken to indicate that it combined with thiol groups of two different reactivities. These authors also found that the energy required for membrane transfers as judged by the Q_{10} of the inhibited transfer rate was unaltered after treatment with NEM, thus ruling out any general membrane effect as

responsible for the inhibition of glucose transfer.

Using aromatic derivatives of NEM, Smith & Ellman (1973) have shown that potency is related to lipophyllicity suggesting that these inhibitors bind to the carrier within the membrane.

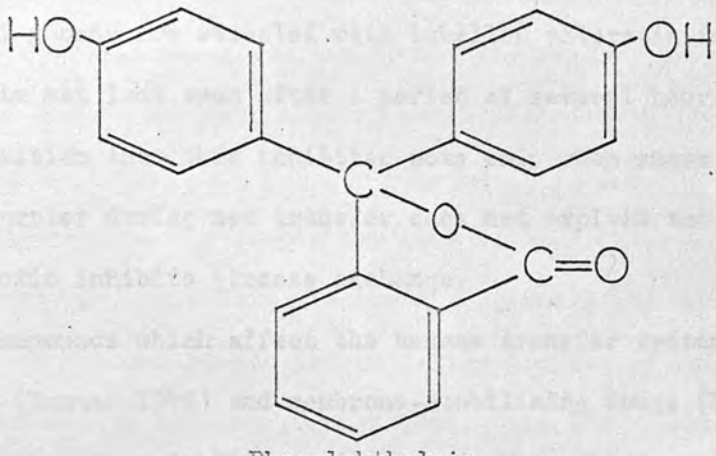
Another important group of hexose transport inhibitors are the diphenols which are reversible and have a competitive action on carrier transport. The inhibitory effect of phlorhizin on glucose transfer in erythrocytes was first observed by Wilbrandt (1947) but he and Rosenberg (1950) found that unlike other tissues the erythrocyte was more sensitive to the aglucone, phloretin and its polymeric form, polyphloretin phosphate. This was confirmed by Sen & Widdas (1962b) who gave values of 1.5×10^{-4} M, $1,5 \times 10^{-6}$ M and 2.4×10^{-6} M for the half-saturation constants of phlorhizin, phloretin and polyphloretin phosphate respectively.



R = H : Phloretin , = Glucose : Phlorhizin.

An investigation by Rosenberg & Wilbrandt (1957b) in which the hydroxyl groups of phloretin were blocked by methylation showed that interference with the phenol group reduced the potency a thousandfold while methylation of the phloroglucinol hydroxyls reduced the activity to about 5%. In a complementary study LeFevre (1959) examined the separate halves of the phloretin molecule which has the hydroxyls intact and showed that they were also almost entirely inactive. He found, however that another group of diphenols typified by stilboestrol, were fractionally more active than phloretin, the 3,3'-di(2-chloroallyl) derivative being some 16 times more effective (LeFevre, 1961). From an examination of the dimensions of these

molecules it was inferred that a spacing of about 10 Å was required between the two terminal hydroxyl groups for in ibitive binding to occur.



Phenolphthalein

A compound related to the diphenols is phenolphthalein which has been investigated by Forsling and Widdas (1968). This has an inhibition constant of the order of 2×10^{-5} M at 37°C and behaves in a similar manner to the other diphenols. Although phloretin is considered to be a competitive inhibitor there is come evidence that its binding may be allosteric. LeFevre (1961) found that Dixon plots (1 /rate v. I) of certain of the diphenols were not linear and Miller (1969) has shown that the data of Sen & Widdas (1962b) for the inhibition of glucose exit by phloretin and polyphloretinphosphate is compatible with an allosteric action.

The inhibition constants for phloretin, stilboestrol and phenolphthalein decrease with temperature (Forsling, 1967) the respective energy changes being 84, 20 and 81 kJ deg.$^{-1}$ mole^{-1}. Thus the behaviour of phloretin and phenolphthalein is similar but stilboestrol suffers much less change. Studies of the uptake of phloretin (LeFevre & Marshall, 1959) stilboestrol and phenolphthalein (Prebble & Widdas, 1969) indicate that these inhibitors are readily adsorbed by the membrane. The uptake of the phloretin and stilboestrol is linear but that of phenolphthalein is of a higher order so that the difference in the energy changes does not appear to be related

to the membrane adsorbtion.

Despite their lipophilic nature these compounds do not appear to penetrate the erythrocyte membrane. Benes et al., (1972) have shown that if red cell ghosts are resealed with labelled phloretin inside them, the labelling is not lost even after a period of several hours. However, their suggestion that this inhibitor acts only when sugar dissociates from the carrier during net transfer does not explain the observation that phloretin inhibits glucose exchange.

Other compounds which affect the hexose transfer system include detergents (Hunter 1965) and membrane-stabilizing drugs (Baker & Rogers, 1972) like the other inhibitors discussed above these would seem to act on the lipid parts of the carrier.

The action of insulin and other hormones is not completely understood since contrary to the finding of most authors (e.g. Sen 1960), Zipper & Mawe (1972) have been able to inhibit part of the transfer system with insulin, its derivatives and certain other hormones. The explanation for the different results probably lies in the very high concentrations of hormone which these workers have used.

The Present Problem.

When beginning this project one of the aspects of greatest concern appeared to be why the half-saturation constant for glucose, determined by various experimental methods, should be so different.

(i). As a contribution to this problem the inhibition of glucose exit by glucose analogues and disaccharides was studied. The competitive inhibition of disaccharides, which are unable to penetrate the red cell, provides a standard for those processes which are restricted for the outer surface of the membrane. The effects of temperature on such inhibitory reactions were compared with those on the inhibitory reactions of penetrating glucose analogues.

(ii) One glucose analogue which was effective as an inhibitor of glucose exit was found to be anomalous in regard to its own penetration through the red cell membrane. This compound 4,6-O-ethylidene-α-D-glucopyranose (ethylidene glucose) was shown not to use the hexose system in penetrating the membrane and is presumed to diffuse through lipophilic regions of the cell membrane.

(iii) These anomalous properties have enabled ethylidene glucose to be used as a probe molecule to investigate separately the competitive inhibition between it and glucose on the inward facing surface of the red cell membrane and have unmasked an asymmetry in affinities of the hexose system as between inside and outside.

(iv) In a collaborative study a reversible but non-competitive inhibition of hexose transfers was observed with a number of psychotropic drugs. The reactions of these drugs with the hexose transfer system in red cells have a number of remarkable features and some of these have been further investigated. To separate membrane effects from direct effects on the hexose carrier the change in the penetration rate of ethylidene glucose was studied. To attempt to determine their mode of action on the hexose transport system net entries of sorbose and exits, entries and exchanges of glucose were all studied.

Although many of the experimental techniques used are common to all these studies, the results can most conveniently be grouped into four separate chapters covering the aspects referred to in the paragraphs above.. The results and conclusions of this work, however, are best discussed together as they provide a cogent set of criteria from which models of the transfer process may be examined.

CHAPTER 2.

METHODS & MATERIALS.

Materials.

The majority of the chemicals used in this work were of 'Analar' grade or better. Those materials of special interest are listed below :-

Sugars:-

Glucose, Sorbose, Raffinose, Trehalose, Cellobiose, Lactose, Glucose-6-phosphoric acid and Sucrose ('Aristar' Grade) were supplied by British Drug Houses Ltd.

Melibiose, Isomaltose, α-Methyl glucoside, β-Methyl glucoside, 2-Deoxy glucose, 3-O-Methyl glucose, 4,6-O-Ethylidene glucose and Tetrahydropyran were from Koch-Light Laboratories Ltd.

Maltose was from May & Baker Ltd.

$^{14}C(U)$-D-Glucose and $^{14}C(U)$-L-Sorbose [34.3 mCi/mM] were obtained from The Radiochemical Centre, Amersham as an aqueous solution containing 3% ethanol as a preservative.

Inhibitors:-

p-Chloromercuribenzoate Na salt was from Sigma Chemical Co.

Phloretin was supplied by Fluka A.G.

1-Fluoro-2,4-dinitrobenzene was from British Drug Houses Ltd.

Chlorpromazine HCl was 'Largactil' from May & Baker.

Scintillator Materials:-

Toluene and Triton-X 100 were supplied by British Drug Houses Ltd.

1,3-Diphenyloxazole (PPO) was from Packard.

2,5-Di[3-phenyloxazoyl]-benzene (POPOP) came from Koch-Light.

Chromatography Material:-

Silica gel G (after Stahl) from Merck.

Preparation of Red Blood Cells.

Human blood was obtained by venepuncture and put into centrifuge tubes containing a trace of dry heparin. The blood was normally used fresh but on occasion was stored for up to three days in the refrigerator in a lightly stoppered tube. When required the cells were washed in 5 ml of phosphate buffered saline and centrifuged at 3000 rpm. for 4 minutes. The buffy coat was removed and the cells washed twice more before being used.

Adult guinea pig blood was obtained from animals under Pentobarbitone Sodium anaesthesia by cardiac puncture. The cells were treated in an identical manner to the human cells.

Solutions.

Cells were normally suspended in a slightly hypertonic phosphate buffered saline. This was made by dissolving 3.12 gm. of sodium dihydrogen phosphate and 8.0 gm. of sodium chloride in almost 1 litre of deionised or distilled water. 7 ml. of a 2N solution of sodium hydroxide was then added and the pH brought up to 7.4 by adding more sodium hydroxide dropwise. The solution was finally topped up to 1 litre with water. The saline was usually freshly made each day but when large quantities were required, ten times concentrated stock solutions of phosphate buffer and of saline were made and diluted as necessary.

The solutions of sugars for the optical experiments were normally of an equivalent concentration to those used by Sen & Widdas (1962a) i.e. a 1.67 M solution corresponding to the 30% glucose solution or a 1:20 dilution of this (83mM). In cases where the solubility of the sugar was insufficient to achieve these, larger volumes of a more dilute solution were used.

For the haemolysis experiments 0.33 M sugar solutions were needed. Since heomolysis is very sensitive to pH changes, these solutions were made up in 30 mM phosphate buffer at pH 7.4.

Solutions of Fluorodinitrobenzene and Phloretin which are relatively insoluble in water were made up by dissolving the material in a minimum of absolute alcohol. Chlorpromazine which is obtained as a 2.5% solution was diluted for the radioactive procedures but was added neat to the cuvette for the optical experiments, 3 µl producing a concentration in the cuvette of 1×10^{-5}M.

All the solutions used in optical experiments were filtered through Whatman no. 50 papers before use to remove dust particles.

For the isotope experiments the sugars were weighed out individually to be added in solid form or dissolved in small volumes to give the required concentration.

Methods.

Two methods were used for the determination of most sugar fluxes in this work. The first was the Ørskov optical method as modified and used by Widdas (1954) and the second a radioactive tracer technique. Both of these proceedures have special advantages, the Ørskov method provides quick, easily observed results but can only be used for net sugar fluxes while the tracer technique is more tedious but provides otherwise unobtainable information about unidirectional fluxes.

The method of Ørskov is based on the fact that the red cell acts as an osmometer and near its normal volume it swells in proportion to the amount of solute that it contains. By means of a suitable apparatus it is possible to follow the change in volume which accompanies sugar transfer. As the cell volume alters so does the amount of light transmitted by a cell suspension, provided the suspension is sufficiently dilute for very few cells to overlap one another in the light path. Since the change in light intensity will be very small and will be of an extended time course it is impossible to obtain useful results by a conventional d.c. amplifier and ordinary photometric device. To overcome these problems Widdas (1953)

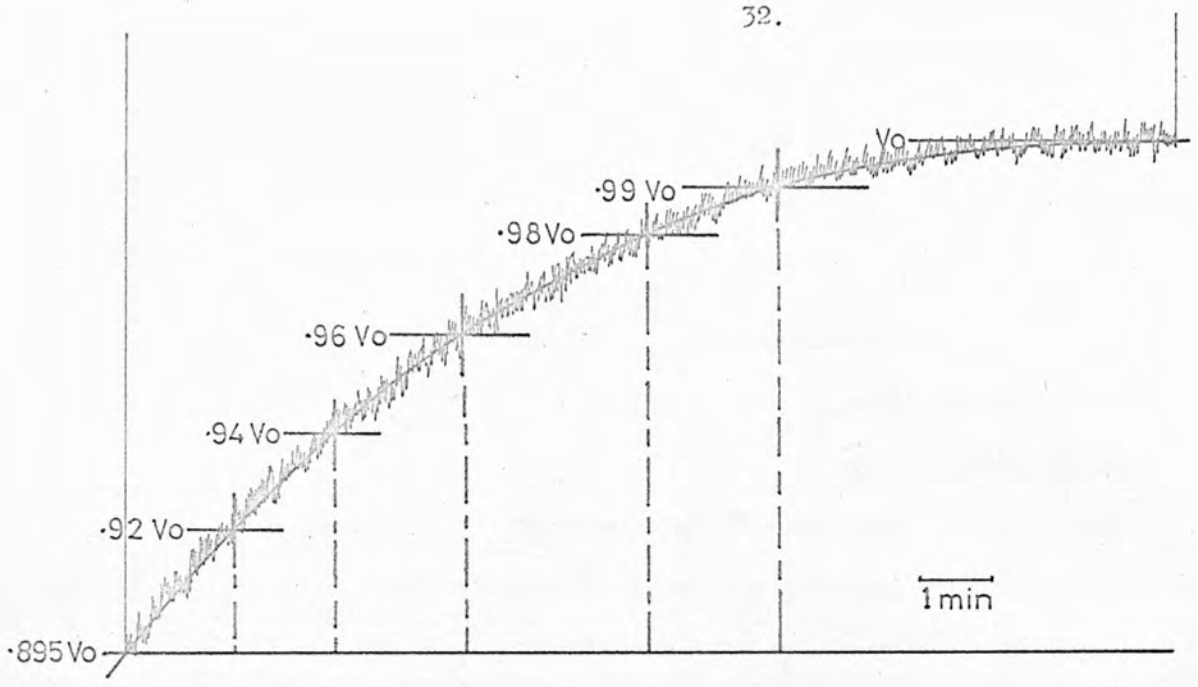


Figure 1.

Entry of 38 mM hexose into Red Blood Cells of 36°C, measured according to Widdas (1954). The rate of sugar transfer may be determined by plotting the times to given volumes against either the near-saturated (F') or diffusion (F) functions given below, depending on the affinity of the sugar.

Hexose Transfer Functions (Widdas, 1954)

Ci	Co	V	F'(C,V)	F(C,V)	Ci	Co	V	F'(C,V)	F(C,V)
0	.1165	.895	-	-	.227	.333	.92	-	-
		.92	.0005	0.27			.94	.030	0.35
		.94	.0019	0.57			.96	.078	0.86
		.96	.0052	1.00			.98	.169	1.76
		.98	.0128	1.75			.99	.265	2.76
		.99	.0218	2.51					
.1165	.227	.91	-	-	.333	.425	.93	-	-
		.92	.004	0.13			.94	.036	0.23
		.94	.015	0.46			.96	.128	0.78
		.96	.034	0.93			.98	.298	1.75
		.98	.071	1.76			.99	.478	2.73
		.99	.112	2.60					

Figures are in Isotonic Units (1 I.U. = 340 mM) and Isotonic Volumes.

designed a differential photometer which used a chopping device to sample the measuring beam and reference beam alternately.

This apparatus produces a sinusoidal signal which can be amplified by a normal a.c. amplifier. By rectifying the output a d.c. signal is obtained which is fed to a pen recorder. The photometer itself uses a single light source to provide two beams of parallel light. One passes through a glass cuvette containing the cell suspension whilst the reference beam passes through a micrometer controlled aperture. The two beams then pass through the chopping arrangement to fall on a photocell. Two models of this apparatus were available. One used mutually perpendicular polaroid filters placed in the light beams and a rotating polaroid disc over the photocell to obtain the necessary alternation between the two beams. The other used an accurately cut rotating disc to achieve the same effect.

In both apparatuses the cuvette is surrounded by a black perspex water-jacket connected to the water-bath in which the suspending media are held. The cuvette is boot-shaped, the light beam passing through the toe of the boot. A stirrer which is mounted on the lid of the water-jacket and fits into the leg of the boot is necessary to prevent sedimentation of the cells.

These apparatuses were used to study sugar entry and exit and also for haemolysis studies.

Procedure for sugar entry. (Widdas, 1954)

3 mm^3 of packed red cells are injected into 21 ml of phosphate buffer saline and allowed to equilibrate until the output trace is level. The aperture on the reference beam is opened by turning the compensator a given amount and 0.5 ml of 1.67M sugar solution is added. Opening the aperture allows for the dilution effect of the sugar and the pen returns to the lower edge of the chart from where it moves across the chart as the entry (Fig.1) proceeds. When the trace has again become level the procedure may be repeated for further entries with the same sample of cells.

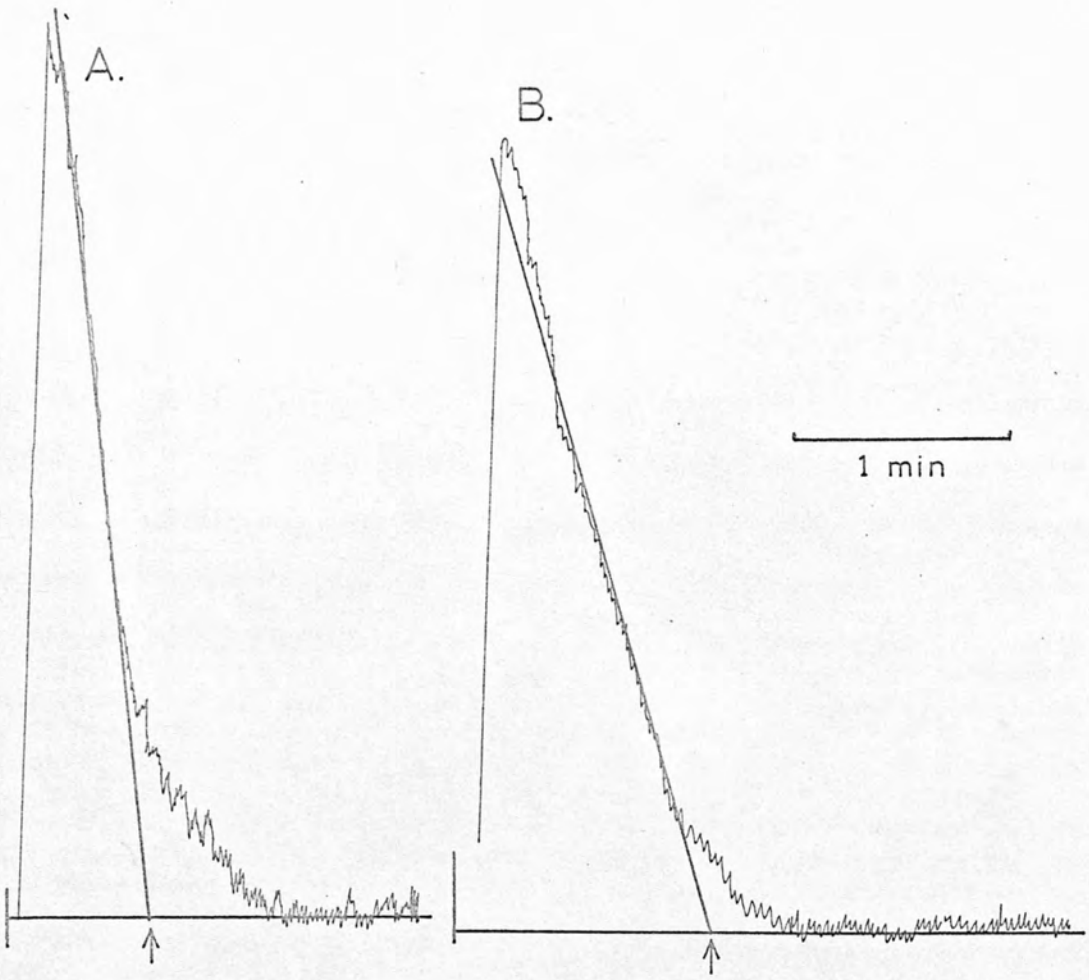


Figure 2.

Exit of 76 mM glucose at 27°C and pH 7.4 into A. 0.7 mM and B. 4.5 mM glucose The traces are measured according to the procedure of Sen & Widdas (1962a). The arrows indicate the respective exit times.

To measure the traces it is necessary to calculate the volume to which the cells shrink, other volumes on the reswelling curve being found by interpolation. The times to reach these volumes are measured as described by Widdas (1954) who has calculated functions relating volumes and times which enable the approximate rates of transfer to be derived according to either of two types of penetration kinetics.

Procedure for sugar exit. (Sen & Widdas, 1962a)

0.2ml of packed cells were suspended in 10.3ml of phosphate buffer saline and 0.5ml of 1.67M sugar and allowed to incubate for about 30 minutes at 36°C. 0.1ml of 2×10^{-3} w/v bile salts solution is added at the end of incubation to make the cells spherulate. After incubation the cells are cooled if necessary and about 0.18 ml. of the suspension injected into 21 ml. of saline or sugar solution in the cuvette. The photometer is set to approximately the right adjustment and when the shutter is opened only a slight adjustment of the compensator is required. This is important at high temperatures as the exit is very fast. The exit is followed until the trace levels out, after which the shutter is closed and the cuvette emptied ready for the next experiment. For exits of sugars with a low half-saturation constant the trace takes a linear form (Fig.2) and the time needed for the cells to empty at the linear rate is determined by producing the linear part of the trace to the base line. The times for exit are plotted as described by Sen & Widdas to derive the half-saturation constant and the maximal rate of transfer. For the exit of sugars with a low affinity the trace takes a curved form and functions similar to those for entry must be used.

Procedure for osmotic haemolysis.

The measurement of solute penetration by osmotic haemolysis predates

the Ørskov technique being first used in 1896 by Gryns. The method makes use of the fact that non-electrolytes which enter the red cell will produce an osmotic gradient as they do so, causing the cell to swell, but since the cell membrane is physiologically impermeable to ions the electrolyte content of the cell will stay constant. If the cell is suspended in a normal saline solution of non-electrolyte its volume, relative to isotonic volume will be given by $V = \frac{1 + S}{1 + C}$, where S is the isotonic quantity of non-electrolyte in the cell and C is the isotonic concentration of non-electrolyte in the medium. It will be seen that in this case the cell volume cannot exceed 1.0 at equilibrium.

If, however, the non-electrolyte is dissolved in water the volume is given by $V = \frac{1 + S}{C}$ which may be rewritten as $V = \frac{1}{(C - S/V)}$, where S is the isotonic cellular non-electrolyte content. By making C isotonic with normal saline, V starts at isotonic volume but as S/V approaches C so V approaches infinity and the cell will swell until it bursts. Rupture of the cell membrane allows the haemoglobin to disperse and the suspension looses its opacity.

Since the change in light transmission that accompanies haemolysis is sufficient to detect by eye, it was necessary to reduce the sensitivity of the apparatus by means of filters and to turn down the gain of the amplifier.

For the experiment 3 mm^3 of cells suspended in 0.25 ml of saline were injected into 21 ml of a 0.33 M solution of non-electrolyte buffered with 0.03 M phosphate buffer. The change in light transmission of the suspension was followed on the chart recorder until complete haemolysis had taken place. It was necessary to calibrate the chart for percentage haemolysis and this was done by suspending one aliquot of red cells in 21 ml of distilled water and then adding 0.19 gm of sodium chloride. This solution was mixed

in the required proportions with a suspension of cells in saline to give the equivalent of 0%, 25%, 50%, 75% and 100% haemolysis. The critical volumes of the cells were also determined by injecting aliquots of cells into solutions of saline or inositol of a known range of tonicities.

From the critical volumes it is possible to determine the percentage of cells which burst when they contain a given amount of solute and by relating this to the haemolysis trace one can work out the rate of entry of the non-electrolyte.

Radioactive tracer techniques for measuring sugar fluxes have been modified by various authors in order to extend their range of application and to improve their accuracy.

The method basically consists of following the flux of a labelled sugar across the cell membrane by determining the number of counts in one compartment of the system, i.e. the cells or the suspending medium, at suitable intervals of time. The problem with this, however, is that it is difficult to separate the cells from the suspending medium sufficently rapidly to prevent the flux continuing and so causing large errors. In this work the transport was halted by adding the cells to an ice-cold, mercury-containing stopping solution and the technique followed was based on that described by Miller (1968a).

Procedure for tracer technique.

The composition of the incubating and suspending media may need to vary with the type of flux being measured but since alterations in volume are undesireable, cells were always suspended in solution of the same tonicity as the incubating medium, further, for exchange experiments the internal and external concentrations of the sugar whose flux was being measured were the same.

Cells are incubated in 5 ml of saline sugar solution at 36°C for at

least 30 minutes and then spun down. 0.3-0.4ml of the packed cells are suspended at zero time in 20 ml of the suspending media which has been placed in a flask surrounded by a water-jacket. The suspension was stirred by a magnetic stirrer and also by working the plunger of an automatic syringe which was used to withdraw 1.0 ml samples at suitable intervals of time, usually 5-10 secs. plus duplicate samples after several minutes (infinite time). On withdrawal of a sample the contents of the syringe were immediately injected into 10 ml of ice-cold stopping solution in a conical centrifuge tube. Full tubes were stored in ice, usually for less than two minutes, until required for spinning. After centrifuging, the supernatant was removed from the pellet of cells and the sides of the tube washed down with a further 2 ml of cold stopping solution and respun. The supernatant was then completely removed and 50 µl of saline added. The pellet of cells was resuspended in the saline using a mechanical stirrer and 1.2 ml of absolute alcohol added whilst stirring. The tube was again spun, this time to settle the precipitated protein, and 1.0 ml of the alcoholic solution was placed in a counting vial containing 10 ml of phosphor. The vials were counted in a Packard scintillation spectrometer for 20 minutes using a preset ^{14}C channel.

Providing the volume of the cells stays constant the sugar will exchange at a steady rate. If the specific activity of the sugar in the cells is N and that in the medium is N_e then at any time t, the transfer of counts will be $\frac{dN}{dt} = k(N_e - N)$, where k is a constant.

Integrating and considering the conditions for entry where at $t = 0$, $N = 0$, $\ln N_e - \ln(N_e - N) = kt$ or $N = N_e(1 - e^{-kt})$.

Similarly for exit of labelled sugar where $N = N_o$ when $t = 0$, $\ln(N_e - N_o) - \ln(N_e - N) = kt$ or $(N - N_e) = (N_o - N_e)e^{-kt}$, where N_e refers to the external counts at equilibrium in this case.

On the basis that the loss or gain of counts by the cells has a negligible effect on the external specific activity because of the small volume of the

cells relative to the medium, the counts due to extra-cellular fluid trapped in the cell pellet will be constant for all samples. By subtracting the equilibrium counts from the other counts before the logarithmic plots are made this source of error will be removed from the results and will not affect the rate constants.

The steady flux $\frac{dS}{dt}$, may be related to k by multiplying by the intracellular sugar concentration in appropriate units.

Procedure for FDNB inhibition (Bowyer & Widdas, 1958)

44 mg of FDNB were dissolved in 11.4 ml of absolute alcohol and made up to 100 ml with buffered saline. 0.2 ml of packed cells were incubated in 5.7 ml of saline and 0.5 ml of strong sugar solution (1.67 M) at 36°C for 30 minutes and then 4.6 ml of the FDNB solution was added to give a drug concentration of 1 mM. The cells were incubated at 25°C for a further 30 minutes at which time half the 5 ml of suspension was removed and centrifuged. The cells were resuspended in 5 ml of 76 mM glucose solution and incubated at 36° again for 1 hour. The remainder of the sample was centrifuged after 90 minutes and similarly treated. The incubated cells were used for glucose exits in the normal way.

Procedure for Thin Layer Chromatography.

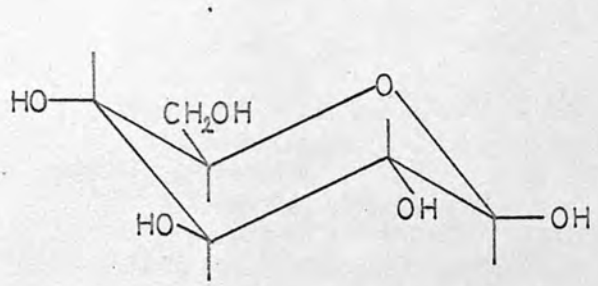
To check the purity of the sugars used, thin layer chromatography was carried out on glass plates 20 x 20 cm coated with Silica gel G (after Stahl) suspended in a 0.1 N solution of boric acid. 30 gm of Silica gel and 60 ml of boric acid solution was used to cover five plates. The coated plates were allowed to dry in the air before heating to 100°C in an oven and were then stored in a desiccator until required for use. Spots of about 1 µl of 30% sugar solution were used and the plates were developed for 30 - 40 minutes with a butanol:acetone:water mixture (4:5:1). On removal from the tank the plates were dried and the spots identified by spraying with

aniline phthalate reagent which caused them to show as light spots under an U.V. lamp. Disaccharides were first sprayed with 0.1 N HCl and allowed to dry at room temperature so that they were hydrolysed before staining.

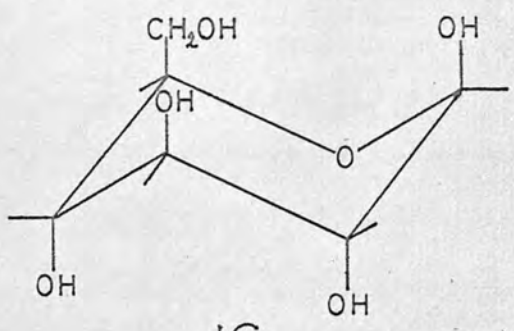
CHAPTER 3.

STUDIES ON THE REACTIONS OF VARIOUS SUGARS WITH THE HEXOSE SYSTEM.

The first step in any facilitated process is the combination of the substrate with a site in the facilitating system. By studying the affinity of the hexose site for various sugars it is possible to collect information which may indicate the size and shape of the site. Early attempts to define a specific pattern for transferred sugars were not conclusive and it was not until LeFevre & Marshall (1958) compared the half-saturation constants of various sugars that such a pattern emerged. It was found that of the eight possible conformations that are stable for a six-membered ring - six boat-shaped and two chair-shaped, the one which was most favoured by the hexose carrier was the conformation known as C1.



C1



1C

The C1 conformation is the one which causes the least strain in the β-glucose molecule since all the bulky groups lie in equatorial positions. Axial groups make this conformation less stable and allow the molecule to adopt other forms. Most sugar solutions, therefore, will normally consist of a mixture of two or more of these different forms in equilibrium.

It is not possible, however, to explain the variation in half-saturation constant purely on the basis of the percentage of sugar present in the C1

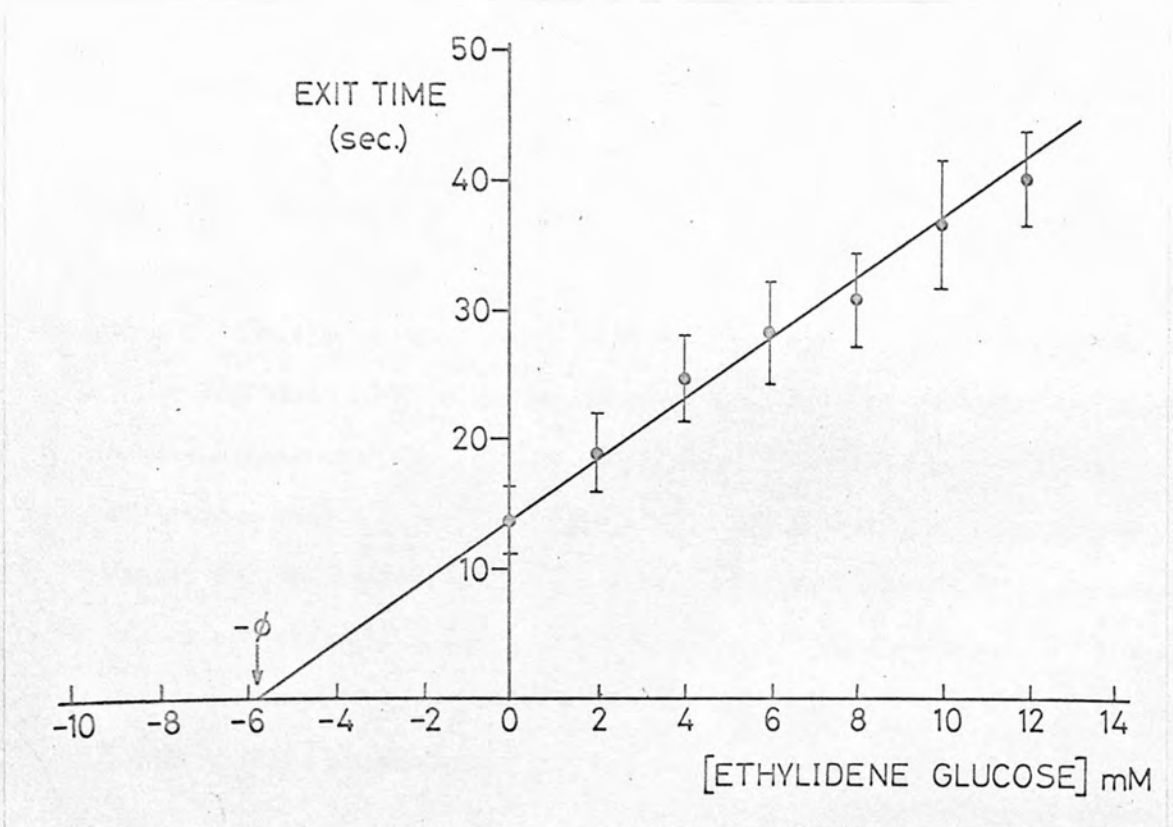


Figure 3.

Means and standard deviations of exit times of 76 mM glucose into increasing concentrations of ethylidene glucose of 36°C and pH 7.4 The arrow indicates the apparent half-saturation constant which must be corrected for the competitive effect of the glucose from the cells.

conformation since 2-deoxy glucose has a half-saturation constant less than that of β-glucose and 4,6-O-ethylidene glucose, which is fixed rigidly in the C1 conformation, has a half-saturation constant that is rather more.

The effect on the sugar affinity of substituting the various hydroxyl groups by hydrogen atoms or methoxy groups has been reviewed in a general fashion by Rosenberg (1961) but he has given no quantitative results so that comparisons cannot be made between the effectiveness of substituents at different positions.

Lacko & Burger (1962) have studied the inhibition due to certain oligosaccharides. They found that non-reducing sugars caused no inhibition of glucose flux and that the effectiveness of inhibiting sugars decreased as the number of saccharide units was raised.

In the present work it was attempted to measure the half-saturation constant of a large number of oligosaccharides and glucose derivatives by the glucose exit procedure of Sen & Widdas (1962a). This method is suitable for this type of study since the results are obtained quickly and easily, and the procedure is readily adapted to different temperatures, so that the determination of temperature coefficients presents no problems.

The effects of the presence of various sugars in the suspending medium over a range of concentrations from 0 mM to 80 mM or approximately 2 x K_i which ever was the lower were examined. The effect was first studied at 36°C but if any inhibitory activity was found experiments were repeated at 27° and 17°C. Determinations were repeated at least three times at each concentration but usually 5 - 10 times.

The K_i was determined by plotting the sugar concentration against the exit time which gives the so called Sen-Widdas Plot seen in Fig.3. The lines were drawn by eye and the apparent inhibition constant, determined from the intercept on the abscissa which is equal to $-K_i'$. This was

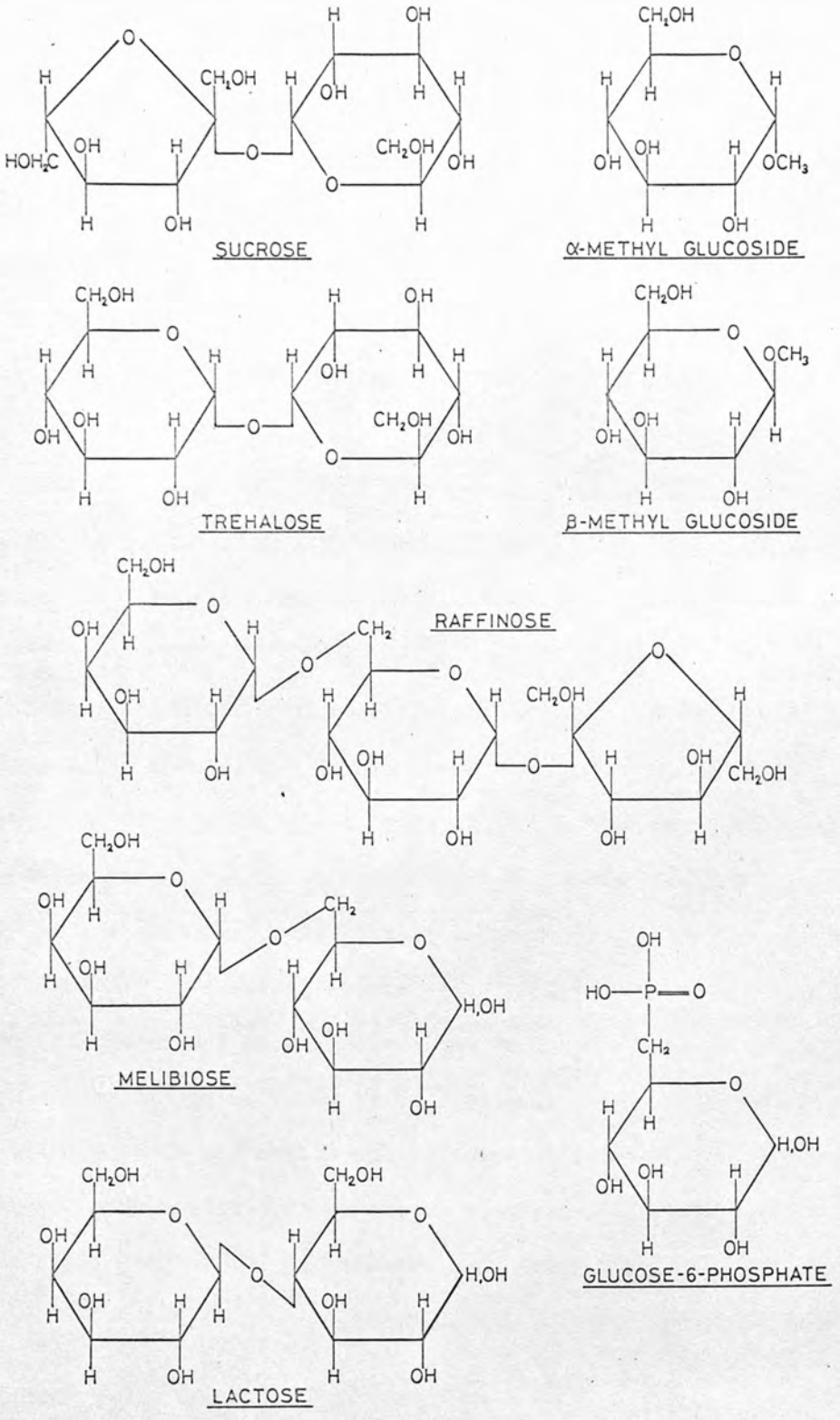


Figure 4.

Structures of Non-inhibiting Sugars.

accurate to within ± 5% but to allow for the glucose that is present in the cell suspension (0.7 mM) a correction of $1/(1 + C/\varphi_g)$ must be made to the value obtained. The necessary correction factors take the following values 0.704 at 17°, 0.785 at 27° and 0.854 at 36°C.

Results

The sugars studied may be divided into two groups, those which show no inhibition of glucose efflux and those that possess some degree of inhibitory activity. Table I. lists sugars in the first catagory.

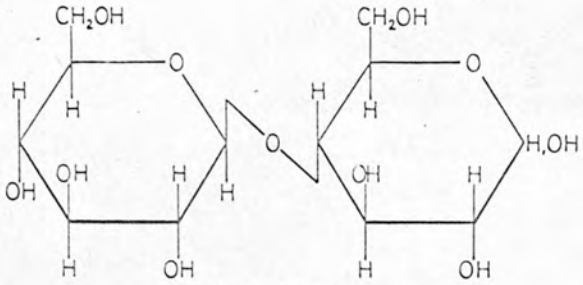
TABLE I

Non-inhibiting Sugars

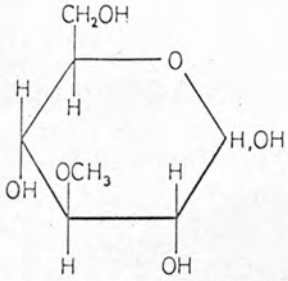
(See Fig. 4.)

Raffinose	Melibiose
Sucrose	Lactose
Trehalose	Glucose-6-phosphate
α-Methyl Glucoside	
β-Methyl Glucoside	

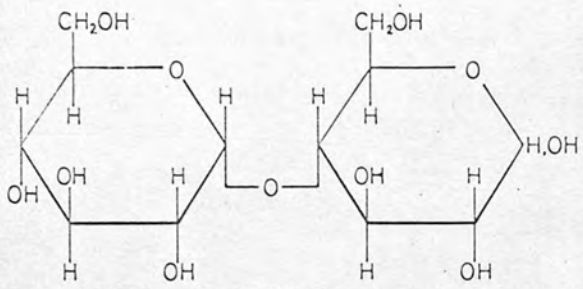
In those sugars on the left of Table I the reducing group on the glucose molecule is blocked but in the sugars on the right, this group is free and the lack of effect must be due to some other cause. Of these sugars, lactose is peculiar in that the rate of exit does not appear to increase with the osmotic effect of high concentration. The osmotic effect of non-electrolytes in the suspending medium gives the Sen-Widdas plot a very slight negative slope if high concentrations of non-inhibiting sugars are being examined but the plot for lactose is perfectly level, suggesting that there may be a fractional amount of inhibition. If this is so the K_i for lactose must be well over 100 mM but the effect might also be explained by a trace of glucose in the solution since all



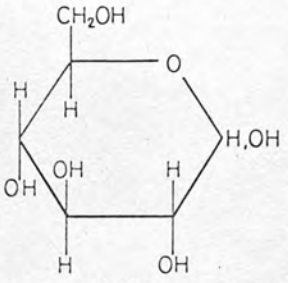
CELLOBIOSE



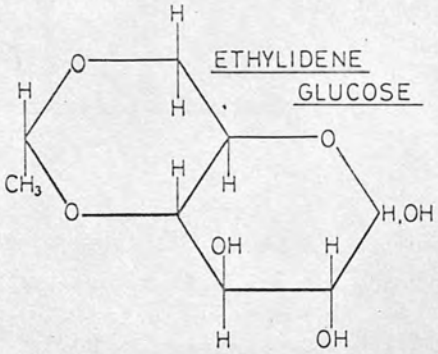
3-O-METHYL GLUCOSE

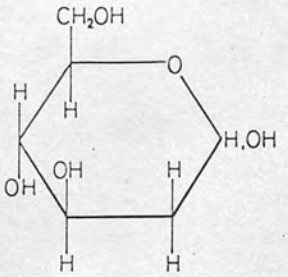


MALTOSE

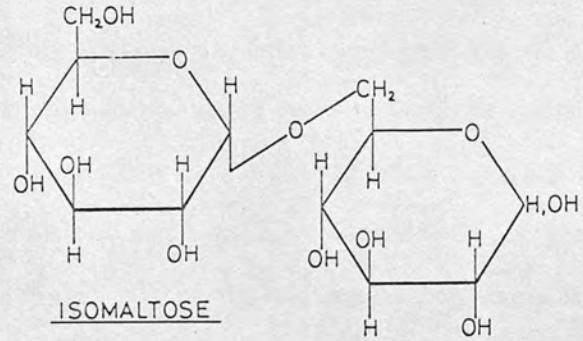


GLUCOSE

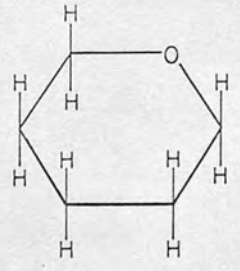




2-DEOXY GLUCOSE



ISOMALTOSE



TETRAHYDROPYRAN

Figure 5.

Structures of Inhibiting Sugars

disaccharides are likely to suffer from some hydrolysis. This problem was specially severe in the case of sucrose and for this reason the very pure 'Aristar' product was used. Hydrolysis may be the explanation for finding of Levine, Oxender & Stein (1965) that 200 mM sucrose causes inhibition of glucose exchange which would appear to be contrary to the present results.

The second group consists of those sugars which cause an increase in the exit time for glucose and so give a Sen-Widdas plot with a positive slope. These sugars and their corrected K_i's are shown in Table II.

TABLE II

Inhibition Sugars

(See Fig. 5.)

Sugar	K_i(36°C)	K_i(27°)	K_i(17°)
Cellobiose	38 mM	31 mM	25 mM
Maltose	26.5 mM	15.7 mM	8.4 mM
4,6-O-Ethylidene Glucose	5.1 mM	3.6 mM	1.9 mM
3-O-Methyl Glucose	5.1 mM	3.1 mM	1.8 mM
GLUCOSE	4.0 mM	2.3 mM	1.1 mM
2-Deoxy Glucose	2.2 mM	1.4 mM	0.8 mM
Isomaltose	-	~ 36 mM	-
Tetrahydropyran	48 mM	59 mM	64 mM

Isomaltose was determined at only one temperature but showed rather unusual behaviour. In initial studies it was found that an isomaltose solution had lost its activity during the course of the experiment. On measuring the traces it was seen that the degree of inhibition had decreased with time indicating a loss of sugar affinity. For this reason a study of the variation of inhibition was undertaken at 27°C. The results

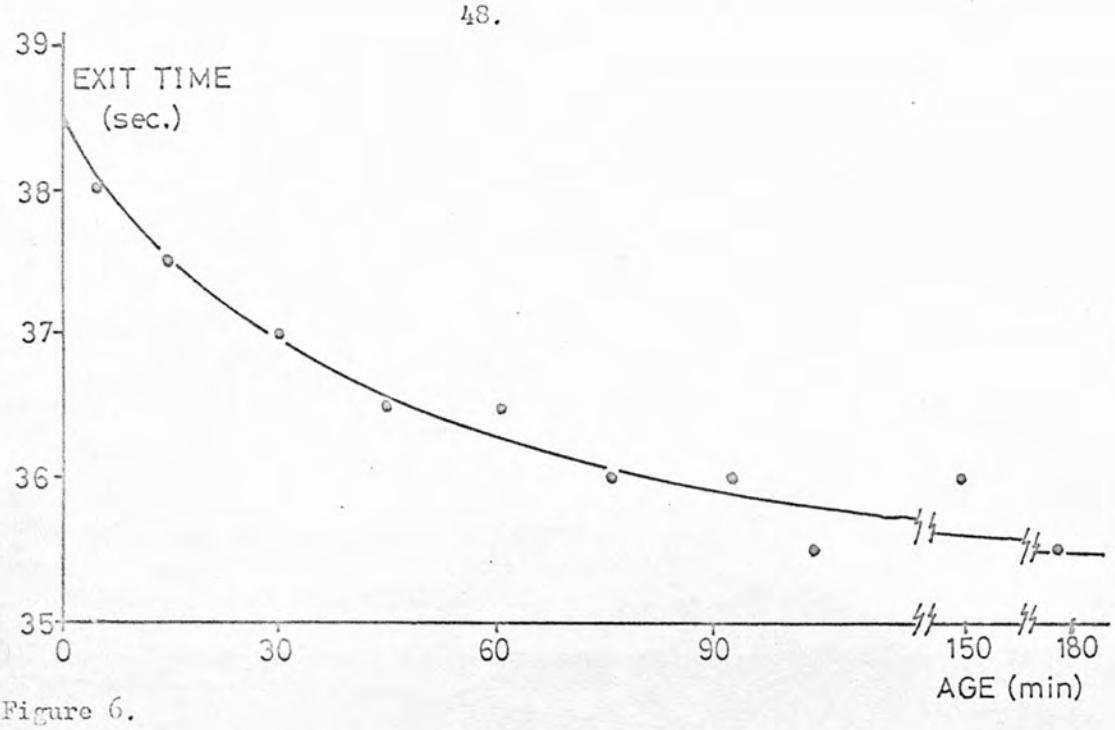


Figure 6.

Decrease in the exit time of 76 mM glucose produced by 3.8 mM isomaltose at 27°C with increasing age of the isomaltose solution. The control exit time was 27 sec.

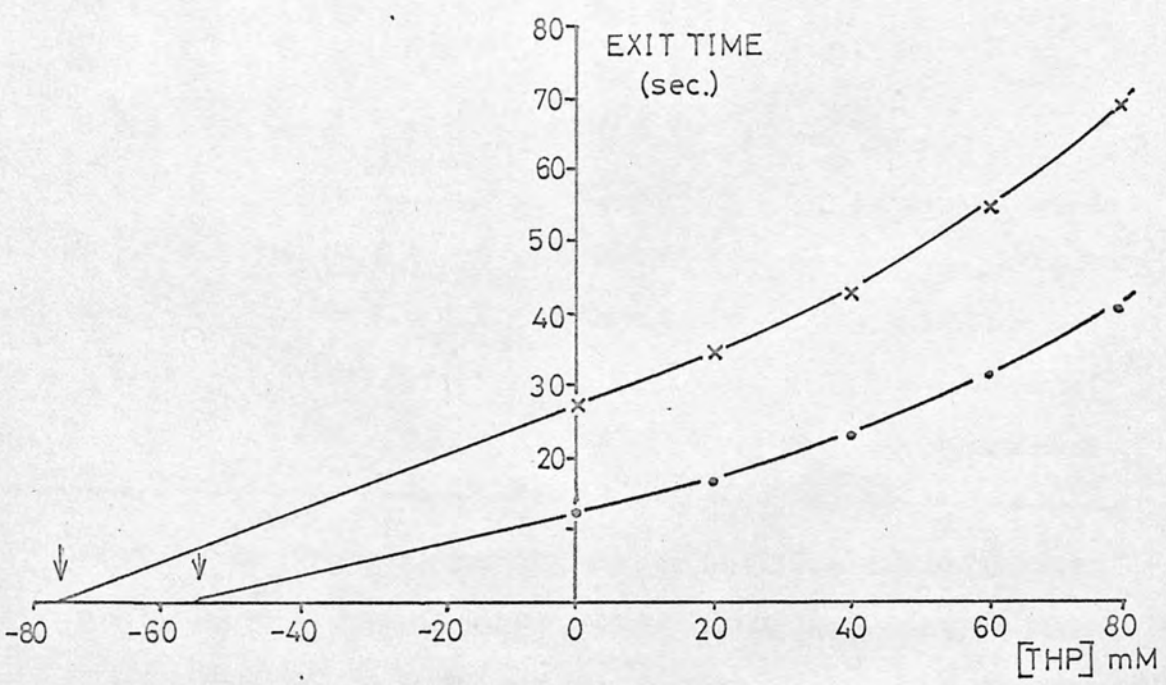


Figure 7.

Exit times of 76 mM glucose into increasing concentrations of tetrahydropyran at 27°C (×) and 36°C (•), pH 7.4. The arrows indicate the estimated inhibition constants.

of this are shown in Fig.6, where the increase in exit time produced by a given amount of isomaltose solution is plotted against the age of the solution. The curve is exponential suggesting a first order reaction, perhaps mutarotation but it levels off to give an increase of $8\frac{1}{2}$ sec. at infinite time. This is due to the presence of a trace of glucose in the sample used to obtain this set of results. In an earlier experiment with a different sample no permanent increase in exit time was observed. Taking the effect of this impurity into account it is calculated from the intercept on the ordinate of the graph that the inhibition constant of the active form of isomaltose at 27°C is about 30 mM.

The tetrahydropyran molecule represents a complete 'deoxy' pentose and it was thought that it might give some indication of the hydrogen bonding potential of the oxygen atom in the pyranose ring. Unfortunately this substance is difficult to work with being relatively insoluble and requiring high concentrations to demonstrate any effect. Sen-Widdas plots for this compound are curved (Fig.7) and the inhibition seen may be due not to a retardation of the glucose flux but to some other action. Preincubation with tetrahydropyran, however, does not alter the degree of inhibition.

Because ethylidene glucose had not been described as an inhibitor of hexose transfer before, the type of inhibition which it causes was determined by studying the interaction of glucose and ethylidene glucose with glucose exits. The results of this study are shown in Fig.8. A small shift in the glucose concentrations has been allowed for with increasing ethylidene glucose concentration because of the trace (about 4%) of glucose in this reagent. The parallel plots, however, confirm that ethylidene glucose is a competitive inhibitor.

A similar graph of the effect of glucose on the inhibition produced by tetrahydropyran gives a set of non-parallel lines with different intercepts on the abscissa. This is characteristic of a mixed or allosteric type of

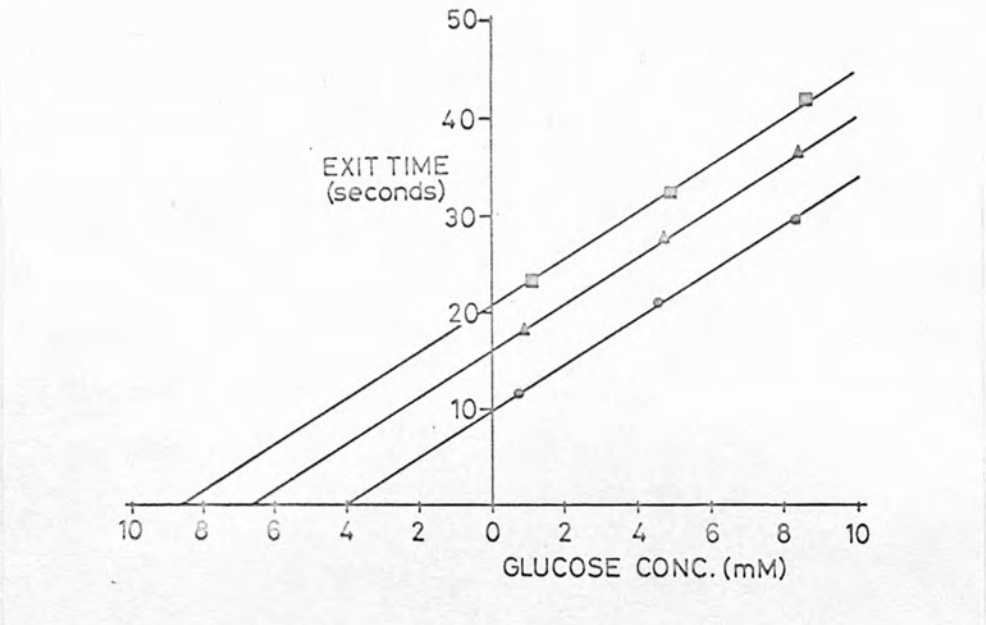


Figure 8.

Competitive effect of Ethylidene glucose on the exit times of 76 mM glucose into low glucose concentrations. Points ● , 0 mM; ▲, 4 mM; ■, 8 mM.

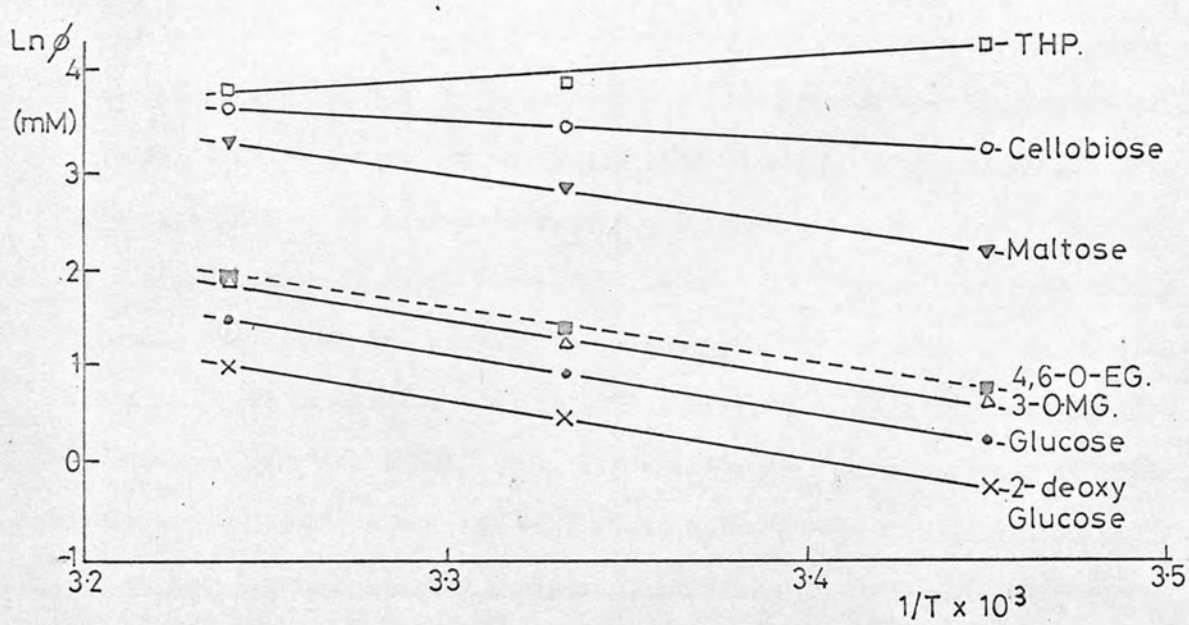


Figure 9.

Arrhenius Plots of the variation of the half-saturation constants with temperature.

inhibitor.

The determination of the half-saturation constants at different temperatures have been displayed in the form of an Arrhenius plot in Fig. 9.

It will be noted that the slopes of the plots for maltose, ethylidene glucose, 3-O-methyl glucose and 2-deoxy glucose are similar to that of glucose giving an energy change for dissociation of about 42 kilojoules/mole. Cellobiose is different in giving ΔE equal to 16.8 kJ/mole although the reason for this is not clear, it would seem more likely that it is due to a change in the equilibrium form of the sugar than a change in the dissociation energy. Tetrahydropyran is also different in that it has a positive slope corresponding to-4 kJ/mole. This type of curve is in keeping with an allosteric inhibiting action and possibly relates to its uptake by the hydrophobic parts of the carrier.

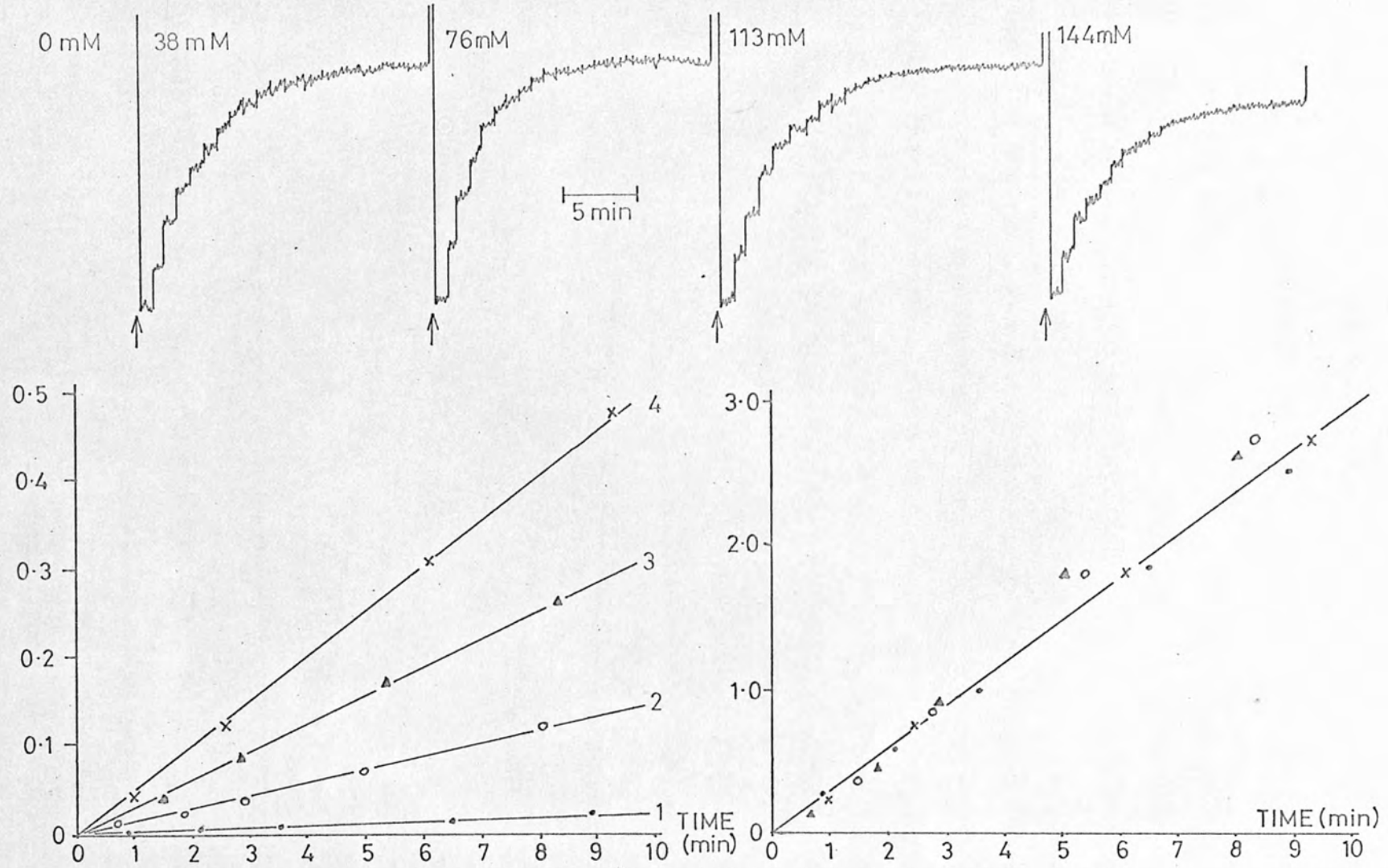


Figure 10. Four consecutive entries of 38 mM Ethylidene glucose plotted against A, near-saturated and B, diffusion functions.

CHAPTER 4

INVESTIGATION INTO THE MODE OF PENETRATION OF ETHYLIDENE GLUCOSE.

In the last chapter mention was made of a glucose derivative - 4,6-O-ethylidene glucose - which, as far as is known, had not been used as a substrate for the hexose transfer system before. A preliminary investigation of its behaviour showed an unexpected anomaly in its kinetics of penetration and thus a further investigation was undertaken to resolve this and to provide more evidence about the properties of what appeared to be a very interesting substance in regard to its interactions with the hexose system and red cell membranes.

4,6-O-ethylidene glucose is prepared by the condensation of glucose and paraldehyde. It is a two-ringed structure consisting of a glucopyranose ring with a second '1,3-dioxan' ring conjoined to it by the 4,5 and 6 carbon atoms of the glucose. Because the C4 and C6 oxygen atoms lie in the same ring the whole molecule is planar and the glucopyranose ring is held in the C1 conformation (see LeFevre & Marshall, 1958).

Because ethylidene glucose is intermediate in size between the single ring of glucose and the two rings of maltose or cellobiose it was originally thought that it might provide some indication of the limiting dimensions for carrier transfer. As was seen in the last chapter ethylidene glucose has, despite its large size, an affinity as close to that of glucose as any other of the commonly used substrates. It thus reacts readily with the carrier and shows no evidence of gross steric hindrance, so that one might expect it to enter the red cell on the hexose carrier system.

Investigation.

In order to determine whether entry into the red cell takes place, the

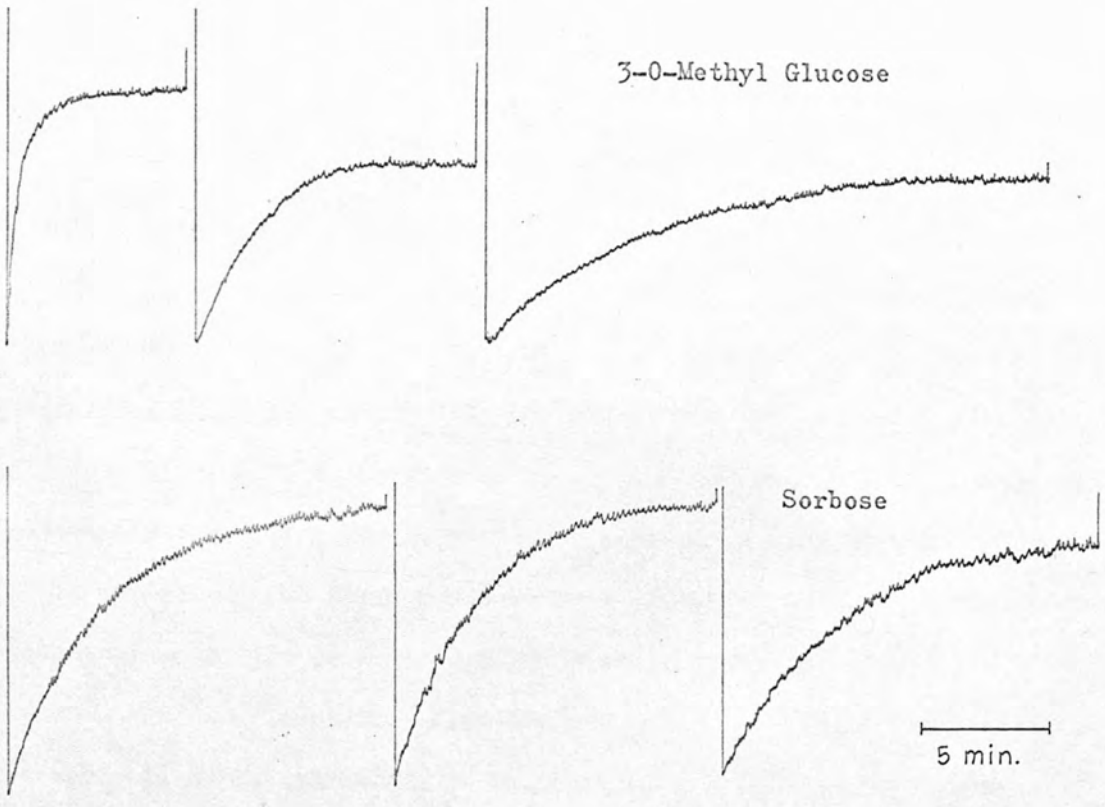


Figure 11.

The entry of consecutive amounts of 3-0-methyl glucose compared to the entry of similar amounts of sorbose. Note the slowing of 3-0-methyl glucose entries due to saturation. The three traces correspond to entries in the [illegible] ranges, 0-38 mM, 38-76 mM and 76-111 mM in each case.

technique described by Widdas (1954) and referred to in chapter 2. was adopted. A typical result from this experiment is shown in Fig.10. Below are two graphs of the result plotted against the functions calculated by Widdas for (i) diffusion type kinetics and (ii) near-saturation type kinetics. It can be seen that ethylidene glucose penetration gives a good fit to the diffusion function with a value for k of 0.41 isotonic units/min. but not to the saturation function. This is the source of the anomaly referred to in the introduction to this chapter.

It was shown by Widdas (1954) that for substrates which react readily with the carrier i.e. possess a low φ, the equation describing penetration takes the near-saturated form whilst for low affinity substrates - high φ -it approximates to diffusion kinetics. An example of each of these types of penetration is seen in Fig.11. The top set of traces show the entry of 3-0-methyl glucose (φ=5.1mM) and it can be seen that the rate of entry decreases as the absolute concentration rises, on the other hand the lower set of traces which are of comparable entries of sorbose (φ = 2-3M) show no slowing down. Thus ethylidene glucose with a half-saturation constant of 5.1 mM should follow the same kinetics as 3-0-methyl glucose but in fact it behaves as sorbose.

There are three possible explanations for this behaviour (i) the hexose transfer system may be more complex than the model of Widdas suggests and the ethylidene glucose molecule being too large to enter on the system for which it has high affinity may nevertheless use components for which it has low affinity and so behave like sorbose. (ii) ethylidene glucose may penetrate on a different system from the hexose system,but one for which it has low affinity, (iii) it may penetrate by diffusion. In order to decide between these possibilities a study of the behaviour of ethylidene glucose in various conditions which affect sugar transfer was undertaken.

The effect of temperature on the rate of entry of ethylidene glucose was determined at 17°, 27° and 36°C. and Fig 12 shows an Arrhenius plot

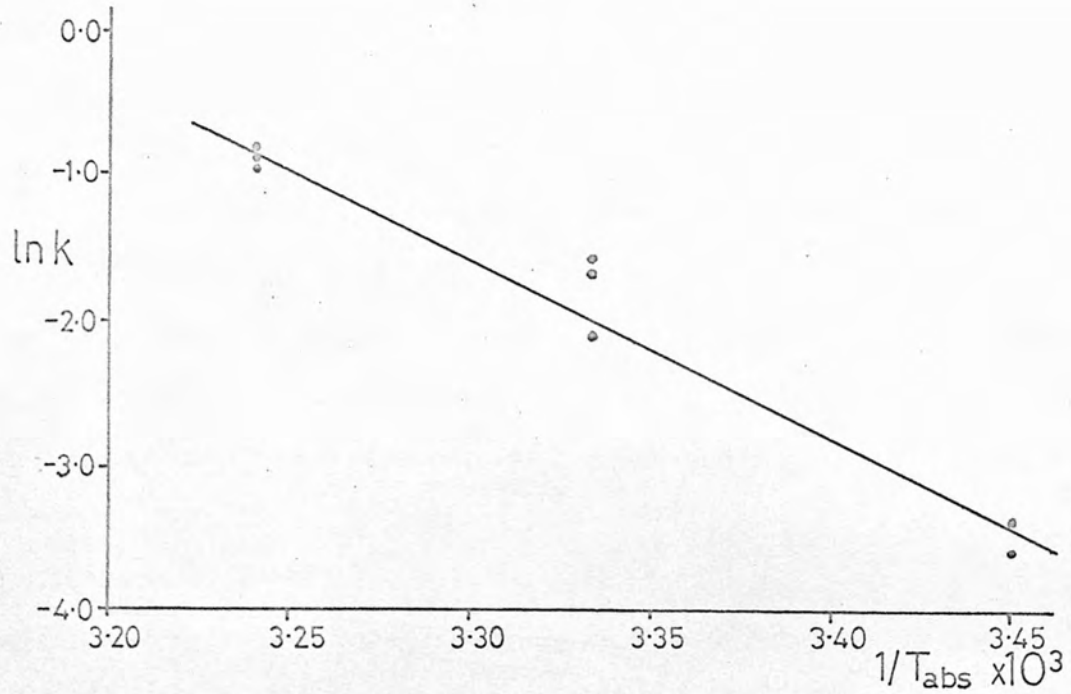


Figure 12.

Arrhenius plot of the rate constants for the entry of ethylidene glucose into human red cells at pH 7.4.

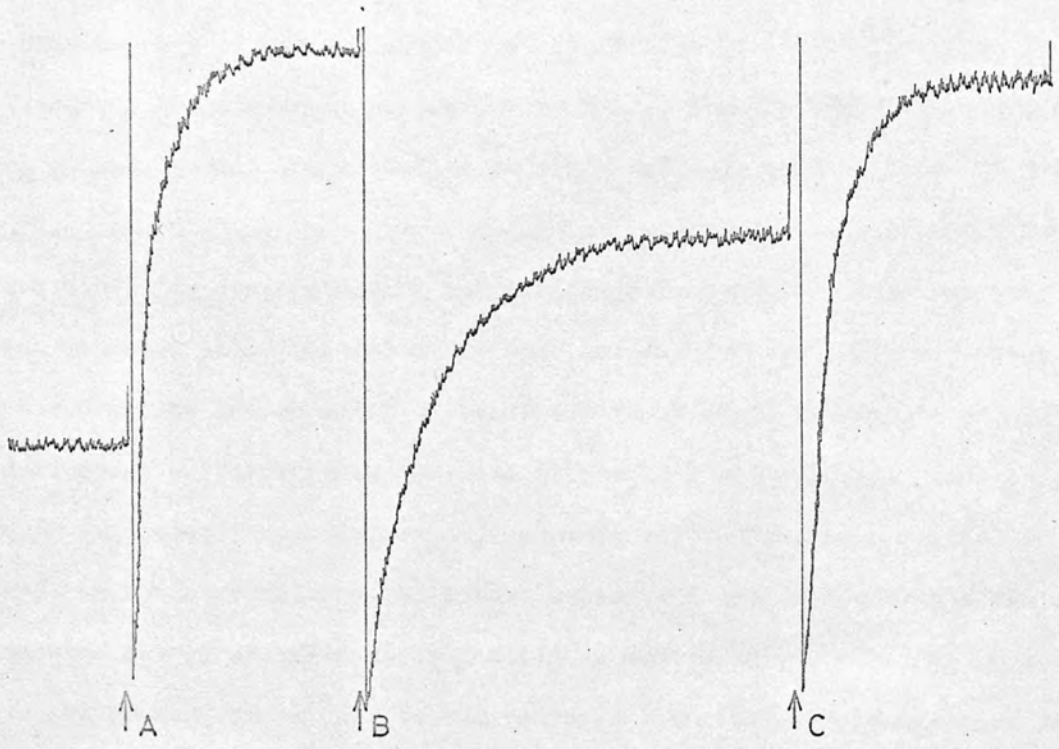


Figure 13.

Inability of Glucose to inhibit the entry of ethylidene glucose. 38 mM ethylidene glucose added at A and again at C, 38 mM glucose added at B.

of the results. The Q_{10} for this process is found to be 4 in contrast to a value of 3 for the process of glucose transfer these Q_{10}'s correspond to 100 kJ/mole and 70 kJ/mole respectively.

If ethylidene glucose penetrates on the hexose carrier it would be expected that its rate would be reduced in the presence of glucose. As can be seen from Fig.13 the presence of 38 mM glucose does not alter the rate of entry of ethylidene glucose. Neither is the rate of penetration of this substance affected by inhibitors of glucose transfer. 10^{-5} M Phloretin and 10^{-5} M para-chloromercuribenzoate both of which reduce glucose entry by 90% have no effect on ethylidene glucose penetration and cells which have been incubated with 1.0 mM FDNB for 100 min. at 25°C so that they show 95% inhibition of glucose transfer still allow ethylidene glucose to enter at normal rates.

Further evidence against the involvement of the hexose carrier was obtained by using adult guinea pig red cells. These do not show glucose penetration by optical techniques but ethylidene glucose penetrates at a rate of 0.64 isotonic units / min. - slightly faster than human red cells.

It is known that glycerol enters the red cell by a process of facilitated diffusion (Stein, 1962) and since ethylidene glucose is similar to glycerol in possessing three vicinal hydroxyl groups, it is possible that both substances use the same system. To test this, the effect of 0.33 M glycerol on the rate of entry of ethylidene glucose was examined but no inhibition could be detected. The affinity of glycerol for its carrier is rather low ($\varphi > 1M$) and it was considered possible that the concentration of glycerol used was insufficient to inhibit ethylidene glucose penetration by a detectable amount. It was, therefore thought necessary to look at the reverse effect. Unfortunately the rate of glycerol entry is much too fast to study by the Ørskov method and it was necessary to use the technique of osmotic haemolysis. The rate of penetration of glycerol into human and adult guinea pig erythrocytes in the presence of ethylidene glucose and the effect of

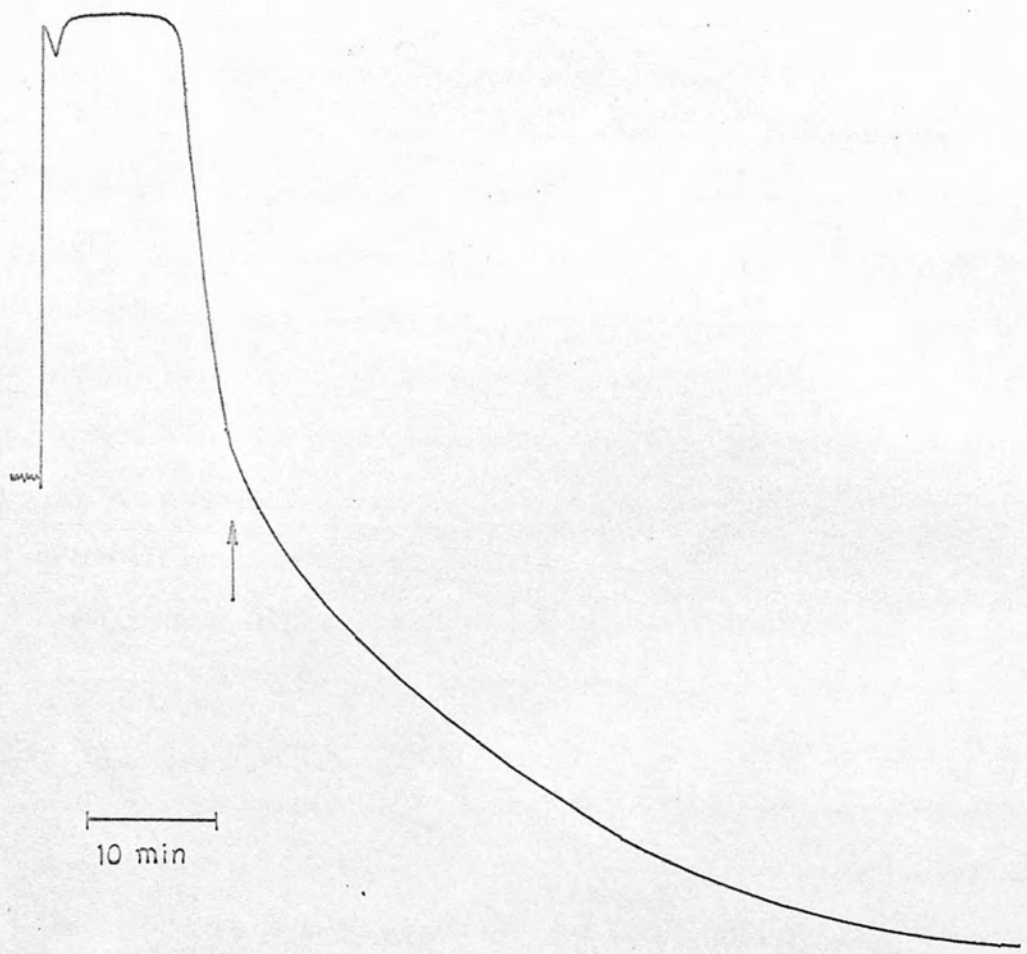


Figure 14.

Development of haemolysis in a sample of red cells suspended in isotonic ethylidene glucose solution (+ 30 mM phosphate buffer). Note the change in rate of the point indicated.

10^{-5} M cupric chloride on the penetration of both these substances was examined. It was found that whilst glycerol was inhibited 60% by cupric ions, ethylidene glucose penetration was unaffected by them and in turn ethylidene glucose produced no effect on glycerol penetration.

An interesting point which arose from these haemolysis studies was the fact that there appeared to be two rates of haemolysis, an initial fast rate and a later, slower rate. This effect, which can be seen in Fig.14 has been interpreted as being due to a leakage of electrolyte from the cell which will, thus, only have its haemoglobin acting as the osmotically active agent. This process is described as colloid osmotic haemolysis and because the concentration of haemoglobin in the cell is fairly small (7 mM) it will proceed at a much slower rate than the more usual electrolyte haemolysis.

If ethylidene glucose does enter the cell by simple diffusion it would be expected to have a partition coefficient considerably larger than that of glucose. An attempt was therefore, made to measure the olive oil:water partition coefficient of this substance by a titrative method but was abandoned as the results were too variable to obtain useful information. Instead a comparison of the solubilities of ethylidene glucose in water and in various organic solvents was made. To find the solubility in an organic solvent 1 gm of ethylidene glucose was shaken for 3 - 5 hours with 20 ml of dry solvent at room temperature and then after centrifugation 10 ml of the mixture were transferred to a weighed specimen jar, allowed to evaporate to dryness and the jar reweighed. To measure the water solubility, ethylidene glucose was added to a known volume of water in 0,1 gm aliquots, the solubility being taken as the last 0.1 gm to be completely dissolved. The final volume of the solution was noted to allow the molar concentration to be determined.

The results are given below, the partition coefficient being obtained from the ratio of the two solubilities.

TABLE III.

Partition Coefficients of Ethylidene Glucose.

Solvent	Saturating Concentration	Partition Coefficient Solvent:Water
Ether	1.7 mM	0.00036
Benzene	1.1 mM	0.00024
Water	4.6 M	-

The partition coefficient of ethylidene glucose for ether:water (3.6×10^{-4}) is of the same order as that for glycerol (3.3×10^{-4}) reported by Collander (1949).

Thus penetration by diffusion is a definite possibility in view of the variety of results described here which suggest that facititated diffusion is not the means by which ethylidene glucose penetrates the membrane. However they do not preclude the possibility that it can be translocated by the hexose carrier to a small extent. If this were so it would seriously affect the detailed interpretation of ethylidene glucose inhibition of glucose transfer and since the unique behaviour of this substance makes several important applications to the study of sugar permeability possible, it is essential to know if this is occurring.

There are two ways in which this can be investigated, the first stems from the prediction of countertransport by Widdas (see Chapter I.p.15)

If ethylidene glucose translocates on the carrier then preloading the cell with it and allowing it to efflux into a solution of labelled glucose would cause an increase in the amount of labelled material inside the cell above that expected for equilibrium. In Fig. 15 we see the amount of radioactive glucose inside the cell during the efflux of 76 mM ethylidene glucose compared with the amounts accumulated during effluxes of the same

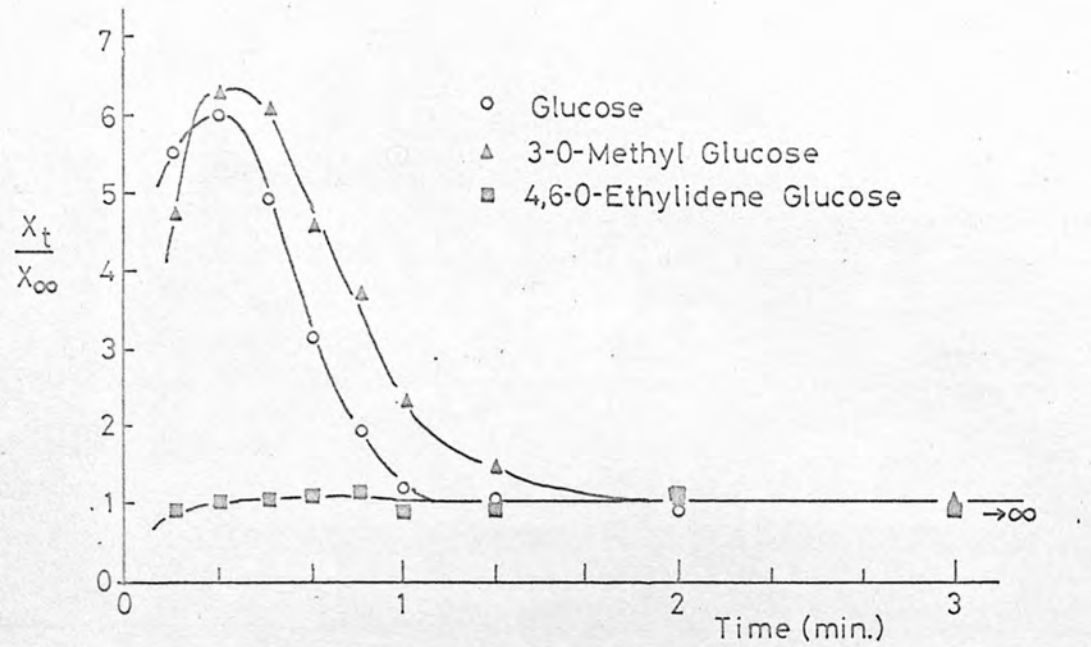


Figure 15. Inability of ethylidene glucose to induce counter-transport. Accumulation of ^{14}C-glucose during the efflux of 76 mM sugar into 4 mM labelled glucose at 27°C plotted relative to the amounts at equilibrium.

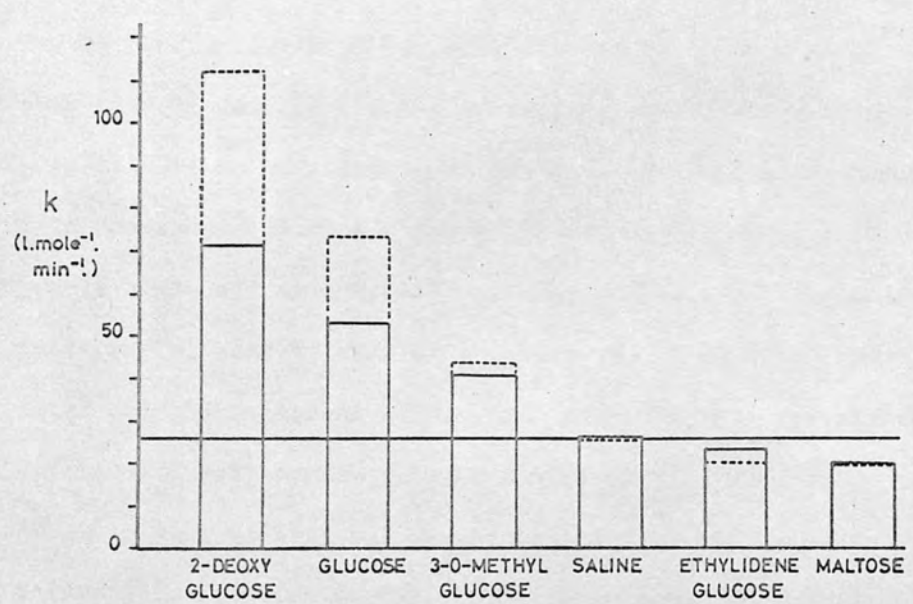


Figure 16. Rate constants for inhibition by FDNB in the presence of various sugars. Results for two different samples of blood incubated for 30 min. with 1 mM DNFB + 76 mM sugar at 25°C.

concentration of glucose and 3-O-methyl glucose. It will be seen that whilst the transported sugars induce a marked counter-transport, ethylidene glucose shows only a steady rise to the equilibrium amount.

A second method of distinguishing between transported and non-transported sugars is based on the observation of Krupka (1971a) that sugars such as glucose, 2-deoxy glucose and sorbose all potentiate the rate of inhibition by FDNB, while cellobiose and maltose which are not transferred partially protect the carrier against this inhibitor. The potentiating action was interpreted as the result of a change of conformation of the carrier during the transfer of a trsnsportable sugar which enabled the FDNB to react, while the effect of the disaccharide was to anchor the carrier to the surface and prevent this change occurring. Fig. 16 shows the levels of FDNB inhibition produced by incubating cells in 76 mM of various sugars for 30 min. at 25°C. It will be seen that whilst 2-deoxy glucose, glucose or 3-O-methyl glucose all potentiate the action of FDNB, ethylidene glucose like maltose, causes a reduction of the effect relative to a sugar free saline control.

It would appear, therefore that ethylidene glucose cannot penetrate the red cell on the hexose carrier and may thus be used with confidence as a probe molecule to study the competitive affinity of the hexose system inside as well as outside the cell.

CHAPTER 5.

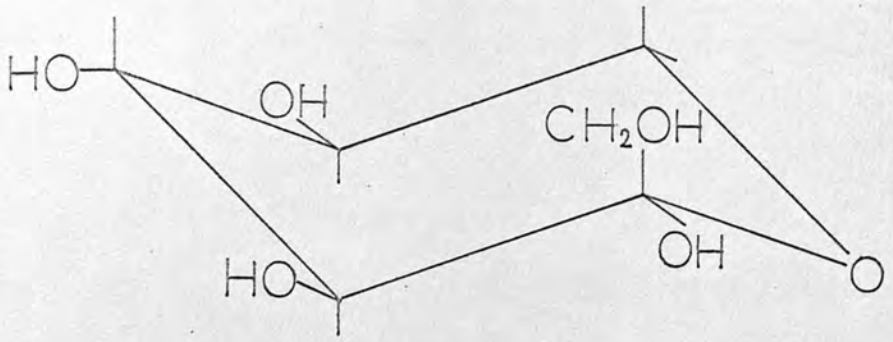
THE EFFECTS OF ETHYLIDENE GLUCOSE ON ISOTOPIC HEXOSE FLUXES.

As indicated in the introduction, much of the evidence against the simple carrier model is derived from studies of the unidirectional fluxes of labelled sugars. It has been shown (Miller, 1968a: Karlish et al.,1972) that the half saturation constants for glucose exchange and for glucose exit into very low concentrations of glucose are much higher than those produced by the Sen-Widdas procedure. Lieb & Stein (1972) have interpreted these differences in terms of their tetramer model and suggest that the exchange and zero-trans procedures give a measure of the half saturation constant of the low affinity units.

The ability of ethylidene glucose to penetrate the membrane independently of the hexose carrier provides a unique opportunity to investigate the inside or outside of the membrane without interference from sugars on the other side. The slow rate of ethylidene glucose diffusion relative to the carrier mediated transport of other sugars permits the maintainance of high concentration of this material on only one side of the membrane during the period required for exchange fluxes to occur. Thus it may be used as a competitive inhibitor of sugar transfer and by this means the relative affinities of the carrier sites on each side of the membrane may be directly measured.

The ability of ethylidene glucose to compete with the carrier without being transferred may be used again to investigate the inhibition of sorbose fluxes.Sorbose has a very low affinity for the carrier and has been used (Widdas, 1954) to measure the half saturation constants of more favoured sugars. Measurements of the half saturation constant of glucose by this means gives a value of 7-17 mM, some two to four times larger than that obtained by the exit procedure. The reason for this variation is unknown and an investigation

of the affinity of ethylidene glucose may give some indication as to whether the cause lies at the surface or inside the membrane.



L-Sorbopyranose

Method.

The basic procedure has been outlined in chapter 2 but small variations had to be made between different experiments to take account of the net movement of material. Since the accuracy of the method depends to a very large extent on the volume remaining constant during the sampling, it is necessary to balance the amount of osmotic material on one side of the membrane by an equal amount on the other. For control exchange experiments there is, of course, no problem but when ethylidene glucose is present, difficulty will arise because of the tendency for this to move down its concentration gradient and it must be balanced by some inert material which will cross the membrane at a similar rate. Malonamide has been used as the balancing material in these experiments but it penetrates the membrane about three times as fast as ethylidene glucose. For this reason, when high concentrations were required as in experiments where the inhibitor is on the inside of the cell, a mixture of one third malonamide and two thirds inositol, which does not penetrate the membrane was used in the outside media. It was found that the ethylidene glucose contained a trace of glucose (4%) and in some experiments this was compensated for by reducing the amount of glucose added.

For sorbose experiments 0.2 ml of packed cells were incubated in 20 ml

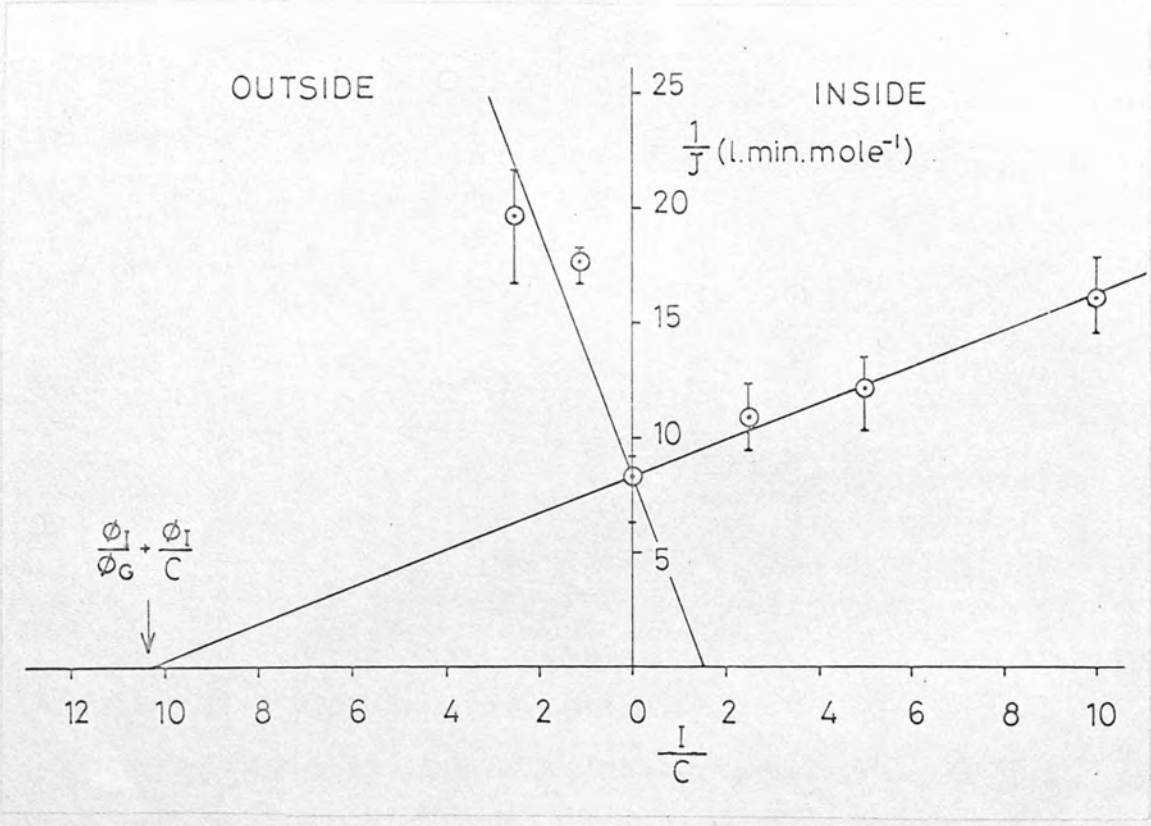


Figure 17.

Reciprocals of the rates of 20 mM glucose exchange at 16°C plotted against the relative ethylidene glucose concentrations with ethylidene glucose inside (points on the right) and outside (points on the left) the cell at 16°C. Points are mean of 3 or more determinations.

of a solution containing 10 mM sorbose and an approximately half-saturating concentration of inhibiting sugar. The suspension was held in a water bath at 36° and at zero-time 2μCi of radioactive sorbose was added. The rate of entry of this into the cells was followed by sampling at intervals of 5 mins. with infinite samples at 60 minutes.

Results

The results of the study of the inhibition of 20 mM glucose exchange fluxes by ethylidene glucose at 16° C is seen in Fig. 17. Exchange fluxes were found to be the same whether the efflux or influx of sugar was measured but for most of the experiments the loss of radio-active glucose from the cell was determined as this was the more convenient procedure. The reciprocal of the rate of transfer of glucose is plotted against the relative concentration of ethylidene glucose inside (+ve slope) and outside (-ve slope) the cell. The intercepts on the abscissa give the respective inhibition constants for the two surfaces. These indicate that whilst the concentration of ethylidene glucose which doubles the glucose exchange time at the outer surface is 30 mM under these conditions, that for the inner surface is 205 mM, a decrease in affinity of nearly seven times. Thus there is a considerable asymmetry between the affinities of the two surfaces for this substance relative to glucose.

In order to measure the actual K_i for ethylidene glucose on the inside of the membrane it is necessary to determine the competitive effect of the glucose present. The intercepts on the abscissa of the above figure may be analysed in terms of the equation for competitive inhibition. Inversion of the equation and equating to zero for the intercept gives :-

$$\frac{I}{G} = \frac{\varphi_i}{G} + \frac{\varphi_i}{\varphi_g}$$

where I and G are the concentrations of inhibitor (ethylidene glucose)

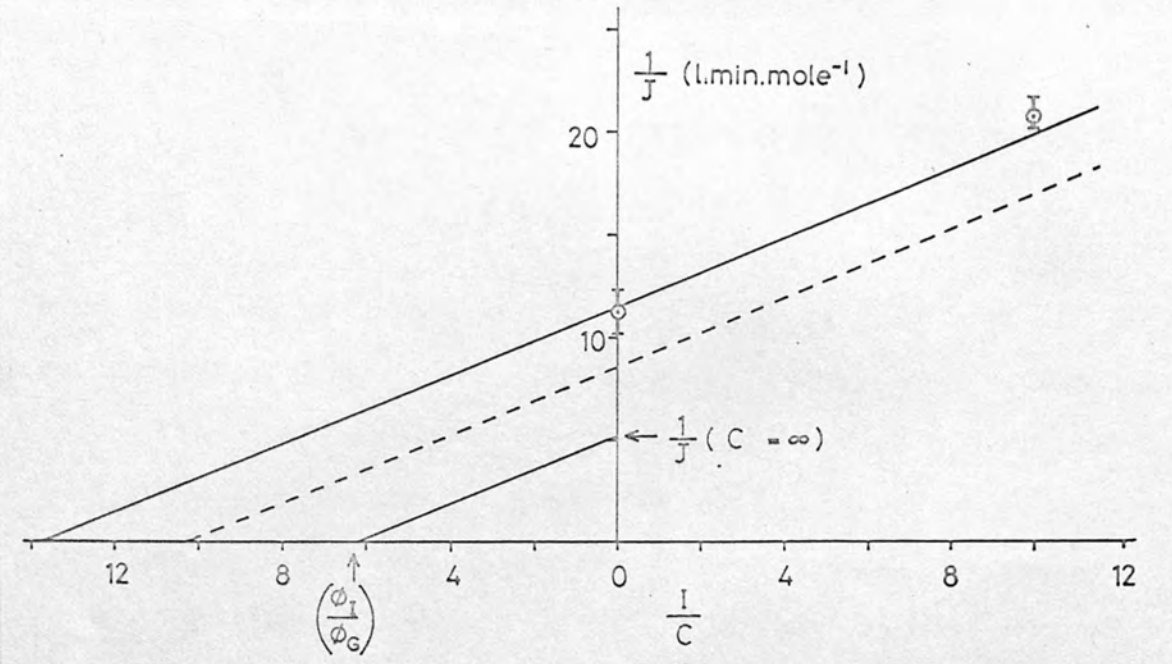


Figure 18.

Reciprocals of the rates of exchange of 10 mM glucose with and without ethylidene glucose inside the cell, plotted against the relative ethylidene glucose concentration at 16°C. The dashed line represents the data for 20 mM fluxes and the solid lines corresponding to 10 mM and infinite glucose concentration are drawn parallel to it. The intercept of the latter line gives the ratio of the half-saturation constants inside the cell.

and glucose respectively and φ_i and φ_g are their half-saturating concentrations at the surface under consideration.

If the above experiment is repeated using a different concentration of glucose, two more intercepts may be obtained which differ from the corresponding intercepts above by a factor of:-

$$\Delta \frac{I}{G} = \varphi_i \left(\frac{1}{G_2} - \frac{1}{G_1} \right).$$

Thus φ_i for each surface may be determined, and in Fig.18. the results of a study at 10mM are compared with those at 20 mM for the inner surface of the membrane. These indicate that the inhibition constant for ethylidene glucose inside the cell is of the order of 70 mM.

The data for the uninhibited glucose exchanges can be fitted to a Lineweaver-Burk plot to give the parameters for this type of transfer and as mentioned in the introduction both the half-saturation constant and the maximal exchange flux are greater than the corresponding parameters for net transfer. It is possible, therefore, that the exchange half-saturation constant for glucose which is of the order of ten times larger than that determined by the Sen-Widdas procedure might, perhaps, be related to the affinity of the inner surface of the membrane.

In order to test this idea the half-saturation constant determined from the intercepts in Fig. 18. may be compared with that determined directly from the uninhibited results. Since the slopes of the lines for the data at 10 mM and 20 mM glucose are the same, a parallel line may be drawn from a point on the ordinate equal to the maximal rate for uninhibited glucose exchanges. This corresponds to the rate of transfer at an infinite concentration of glucose ($1/G = 0$) and will intercept the abscissa where :-

$$\frac{I}{G} = \frac{\varphi_i}{\varphi_g}.$$

This gives a value for φ_g equal to that determined from the uninhibited

glucose exit. Table V shows some preliminary determinations of this parameter (determined by sorbose inhibition) for various sugars compared with the values obtained by the Sen-Widdas procedure.

TABLE V

Inhibition constants for Sorbose Transfer

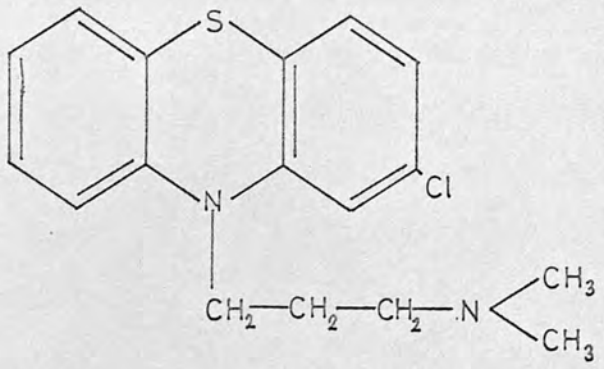
Sugar	φ sorbose	φ S.W.	Ratio
2-Deoxy glucose	5.8 mM	2.2 mM	2.6
Glucose	8.7 mM	4.0 mM	2.2
3-O-Methyl glucose	13.2 mM	5.0 mM	2.6
4,6-O-Ethylidene glucose	8.0 mM	5.1 mM	1.6
Control exchange rate at 36° C		0.32 ± 0.03 min^{-1}	

It will be seen that the values for the inhibition of sorbose transfer are markedly different from those for glucose exit, being about 2½ times greater for the three transported sugars and 1½ times greater for ethylidene glucose. The value for glucose determined here is within the range found by Widdas (1954) and the anomaly is not due to uncertainties inherent in sorbose penetration measurements by the optical methods nor approximations in the analysis, since this procedure is based on a direct measure of the amount of sugar entering the cells. An alternative explanation which takes into account differences in the mechanism of transfer must therefore be sought.

CHAPTER 6.

THE EFFECTS OF CHLORPROMAZINE ON THE HEXOSE TRANSFER SYSTEM.

Chlorpromazine (CPZ) is a phenothiazine derivative with the following structure:-



2-Chloro-10-[3-dimethylaminopropyl]-phenothiazine.

It is of considerable importance in the treatment of mental illness as a non-hypnotic depressant. Its action is believed to be on the afferent pathways of the reticular formation causing a reduction in the flow of arousal stimuli to the cerebral cortex.

It was thought that the effect of CPZ may be mediated through an interference with the penetration of glucose into the brain cells. Because of the similarity between transport processes in these cells and erythocytes a study of the action of phenothiazine derivatives on the glucose permeability of red cells was made (Baker & Rogers, 1972). The results of this study were particularly interesting in regard to the action of chlorpromazine on glucose exit fluxes. These were subject to a complex pattern of inhibition and acceleration and further research was undertaken in an attempt to explain the changes in transfer rate that were observed.

Chlorpromazine has a great many actions on the cell membrane, one primary action being that of increasing the membrane stability to hypotonic

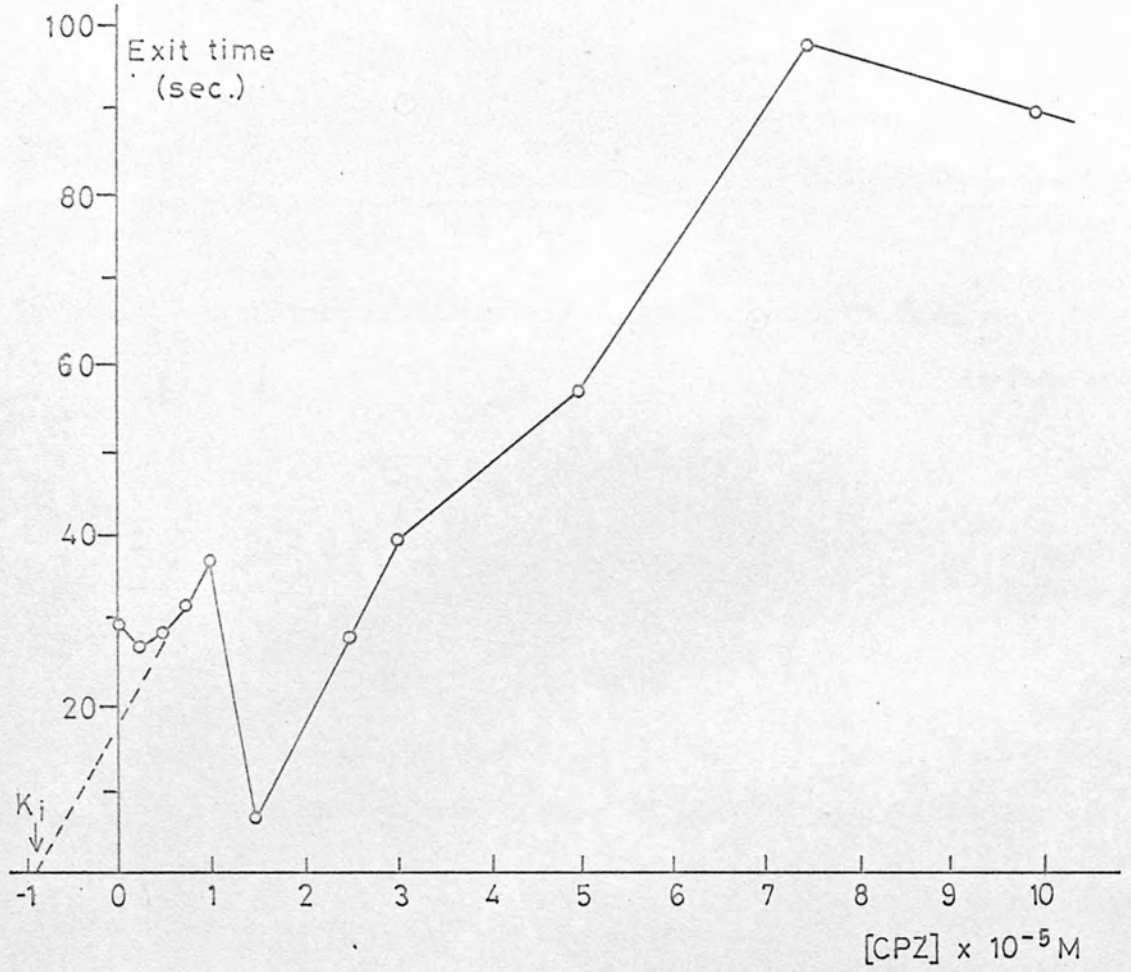


Figure 19.

The effect of Chlorpromazine on the exit time of 76 mM glucose at 36°C. Points are the mean of 3 - 12 determinations. External glucose concentration was 4.5 mM.

haemolysis. This is associated with an increase in the area of the cell membrane which appears to be due to a loosening of membrane structure since as the drug concentration is raised above protective levels, approximately 2×10^{-5}M (Kwant & Seeman, 1969a) lysis occurs. This general loosening of the membrane is also probably the reason for the increase in passive permeability which has been observed (Seeman et al. 1970; Baker & Rogers,1972). In addition to these effects CPZ causes the release of membrane bound calcium, (Kwant & Seeman, 1969b) but whether this is due to a replacement of the ion by a positively charged drug molecule or just a general effect of membrane alteration is not clear.

The importance of these actions in the effects of CPZ on the hexose transfer system is unknown. Alterations in surface charge or in membrane density would almost certainly affect the behaviour of sugar transfer, however, the complexity of this behaviour as shown in Fig. 19 redrawn from Baker & Rogers' (1972) paper is clearly difficult to ascribe to specific effects. This does not, however, preclude the use of this substance as an investigative material to elicit further information on the process of hexose transfer, and an attempt has been made to measure different types of sugar fluxes in its presence.

Methods.

The procedures for the determination of sugar fluxes were the same as used elsewhere but CPZ causes alterations in cell behaviour which are variable, thus making the measurement of results difficult and large numbers of determinations must be made if congruent results are to be obtained. Another difficulty arises from the fact that CPZ is very strongly absorbed by the red cell. The whole cell/saline partition coefficient for CPZ is 27 at 24°C (Rogers, personal communication) and this means that an appreciable fall in concentration can occur when cells are added to a drug solution. For the optical experiments where 0.003 ml of cells were suspended

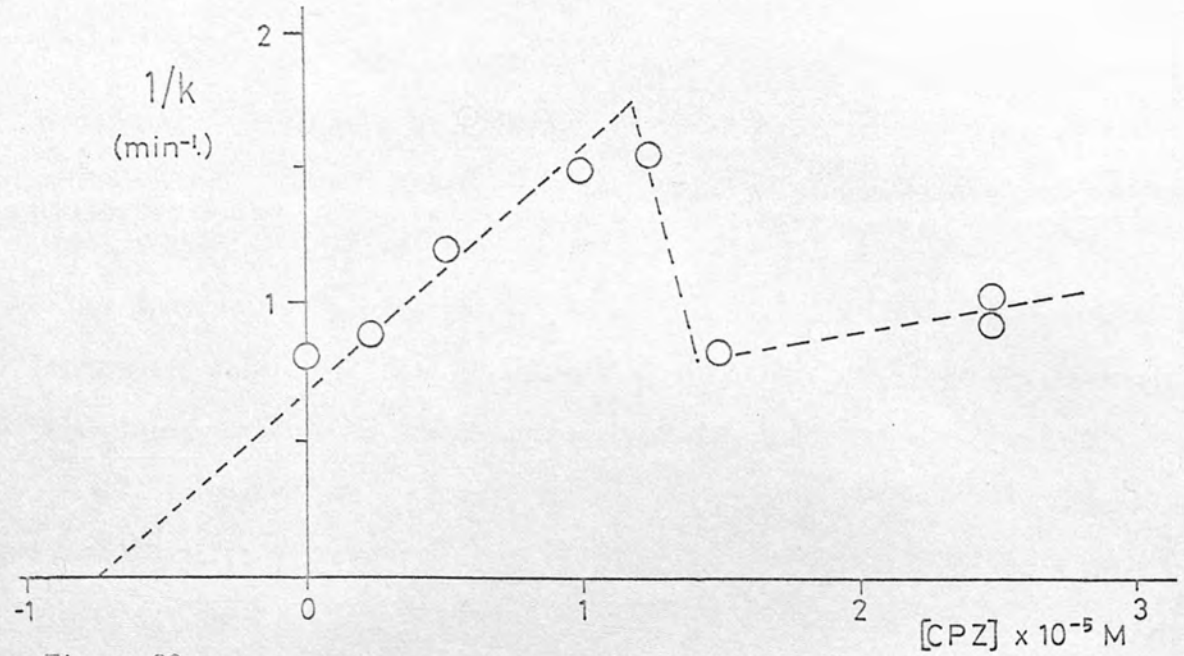


Figure 20.

Effect of Chlorpromazine on the reciprocal of the rates of entry of 38 mM glucose into red cells at 36°C.

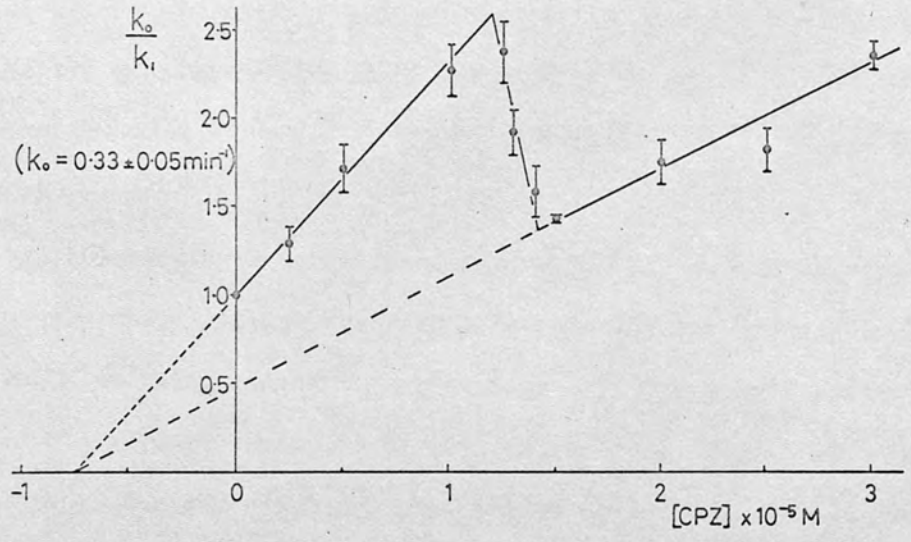


Figure 21.

Normalised reciprocals of the rate of entry of sorbose at 36°C plotted against Chlorpromazine concentration. Points are mean of 3 determinations.

in 21 ml of solution this drop in concentration would be small but in radio-activity experiments where 0.5 ml of cell are incubated in 4.5 ml of solution the effect could be considerable. Therefore in these latter experiments the cells were preincubated twice in 100 ml of drug solution at the required concentration before incubation proper. This brought the effective concentration to within 1.5% of that intended.

Results

As stated in chapter 3. the glucose exit technique is ideal for the rapid examination of inhibitory activity, and for this reason was used in the published work. The glucose entry procedure is less useful since the individual parameters of transfer cannot be determined, however, a number of glucose entries were performed in the presence of CPZ at 36°C. The degree of inhibition relative to a control entry determined on the same sample of blood is shown in Fig. 20. The biphasic effect appears to be present but the cells were unstable in the conditions used in these experiments and very few useful results were obtained. A line may be drawn through the points of inhibited entries at low CPZ concentrations to give the same intercept on the abscissa (0.75×10^{-5} M) as that found for the inhibition of glucose exits at this temperature. Since this graph is analogous to an enzyme-inhibitor plot the intercept is taken as a measure of the K_i for the drug-carrier interaction. Fig. 21 shows the rate of entry of sorbose in the presence of CPZ at 36°C. Sorbose has a low affinity for the carrier and in these experiments gave more stable traces than glucose. The transfer rate shows the same biphasic changes with CPZ concentration as glucose transfer, but the acceleration of transfer is lacking, the flux never exceeding control values. A line may be drawn through the first part of this graph to give the same intercept on the abscissa as the glucose entries while a line drawn through the latter results also intercepts at the same point but the slope decreases as the concentration is increased, suggesting that saturation of the carrier by the drug is occurring.

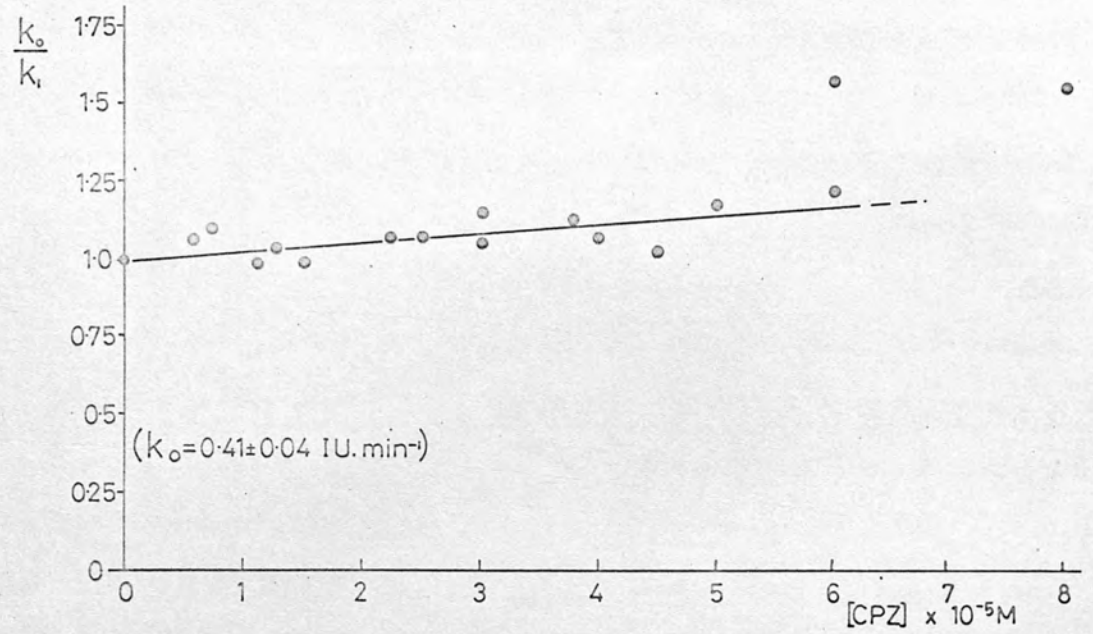


Figure 22.

The effect of Chlorpromazine on the reciprocal of the rate of exchange of 20 mM glucose at 16°C.

The acceleration observed in glucose exits commenced at a drug concentration between 1.0 and 1.5 x 10^{-5} M and the rate returned to the control values at 2.5 x 10^{-5} M. The greater stability of sorbose entries permitted this change to be more closely studied and it was found that at 36°C the initial inhibition was reversed at a drug concentration of 1.25 x 10^{-5} M and the effect lasted over a range of only 2 μM, i.e. to about 1.45 x 10^{-5} M. The second phase of sorbose inhibition, besides being consistant with a line from the same intercept as the first phase, appears to have a slope that is half as great suggesting that this relates to a process which (on the basis of the intercepts on the ordinate) would have had a rate twice that of the control. Thus although this cannot be directly demonstrated because of the concomitant inhibition, it would seem that in the presence of high concentrations of CPZ the carrier has twice its normal capacity. If this is so it is clear that the 'doubling' effect must be related to some conformational change occurring over the narrow concentration range referred to above.

This effect may also be related to the exchange flux of sugar which for glucose, is several times faster than the maximal net transfer. In order to investigate this possibility a series of glucose exchange experiments were carried out and the results are seen in Fig. 22. It appears that glucose exchange suffers little inhibition, the rate remaining practically constant at 140 ± 14 m.moles $l^{-1} min^{-1}$ until the drug concentration reaches a value of 3 x 10^{-5} M which is well above the critical concentration range for the 'doubling' effect.

Some of these experiments were performed conjointly with Dr. H.J. Rogers of the Department of Pharmacology, Guy's Hospital Medical School and it is interesting to compare these results with others from this joint study.

The exchange experiment was undertaken at 17°C because of the high rate of glucose exchange and the results were not directly comparable with those

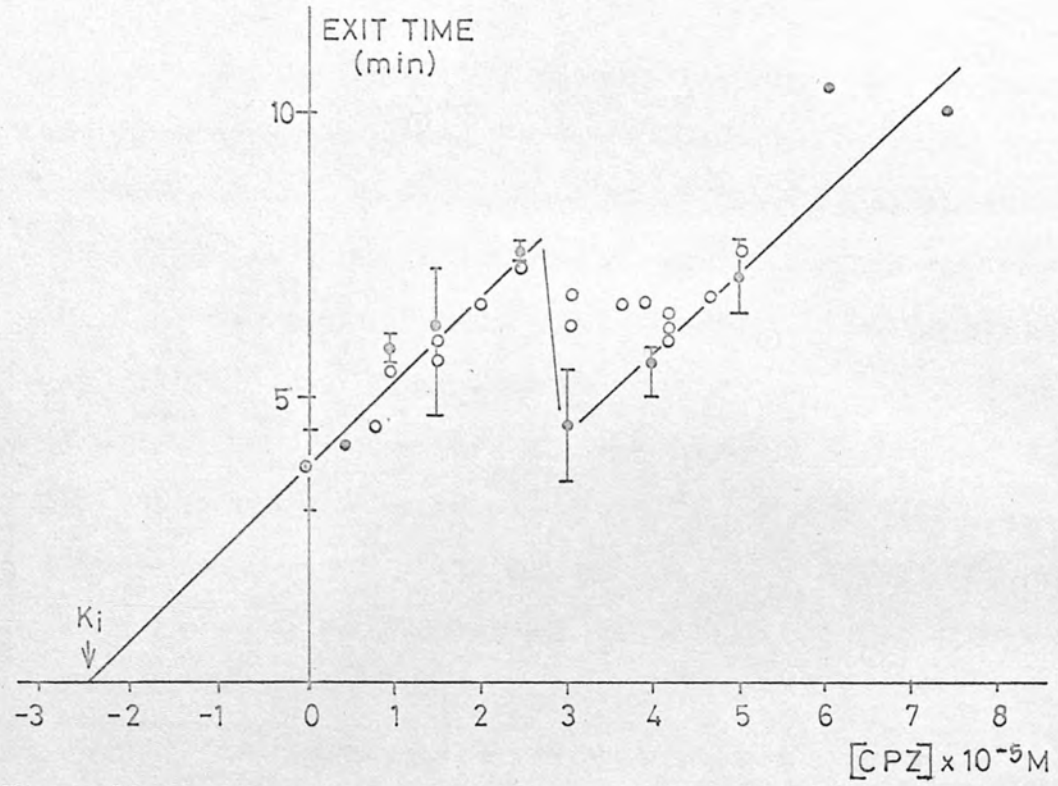


Figure 23.

The effect of Chlorpromazine on the exit time of 76 mM Glucose at 17°C. Solids points, optical experiments; circles, radioactive results.

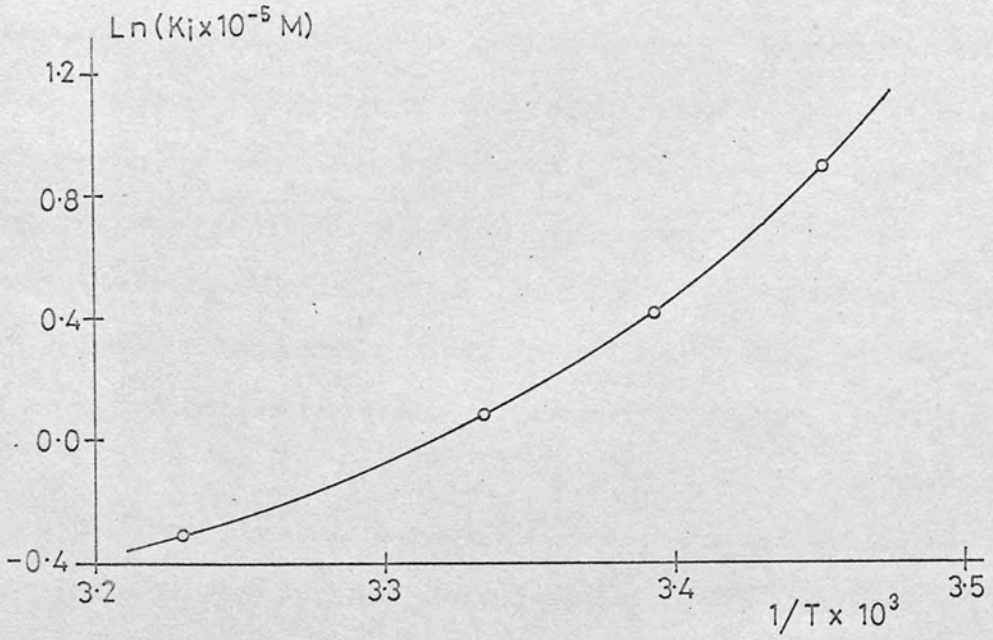


Figure 24.

Arrhenius plot of the variation of the inhibition constant of Chlorpromazine for hexose transfer with temperature.

mentioned earlier. The effect of CPZ on glucose exits was therefore investigated at 17°C using both the optical method and a radioisotope technique. The results of this study are shown in Fig. 23 in which the solid points represent the optical results and the open circles the isotope results. It can be seen that in both cases the ratio of glucose net exits are inhibited two-fold over a concentration range which has a negligible effect on the exchange flux.

The inhibition constant for glucose exits was found to be 2.4×10^{-5} M at 17° and other determinations in this study gave values of 1.1 and 1.5 $\times 10^{-5}$ M at 27 and 22°C respectively whilst a value of 0.75×10^{-5} M was obtained from published work at 36° (Baker & Rogers.1972). An Arrhenius plot of this function can thus be drawn and is shown in Fig. 24. It has a positive slope indicating that dissociation is an exothermic process. An analysis of the thermodynamic constants of the process following that suggested by Kwant & Seeman (1969a) gives values for the free energy, enthalpy and entropy changes which are consistant with hydrophobic bonding but high values for the enthalpy and entropy changes suggest that conformation changes may be produced in the carrier.

The complex action of CPZ still remains to be explained and is unlikely to be so until a full understanding of the mechanism of hexose transfer is available. However, the results suggest certain constraints which must be placed on models of the carrier and these will be discussed in the next chapter.

CHAPTER 7.

DISCUSSION.

At the time when this project was started the kinetics of the hexose carrier mechanism were in some disarray. The rapid growth of radio-isotope techniques had produced a large volume of data which was incompatible with the original carrier model proposed by Widdas but which did not contribute any conclusive facts upon which a more accurate model could be based. This lack of a definite alternative to the simple model is reflected in the plethora of explanations and models which were forthcoming at this time (Levine & Stein, 1966; Naftalin, 1971; Lieb & Stein, 1970; LeFevre, 1972) but although most of these were able to partially resolve the kinetic discrepancies encountered none appeared entirely satisfactory.

As indicated in the introduction most of the discrepancies were centred around differing values for the parameters of transfer, in particular, the values of the half-saturation constants. For this reason the study was directed at an investigation of this parameter in the hope that further information could be obtained upon which a new model could be firmly based.

Structural activity relationship.

Since the attempt by LeFevre and Marshall (1958) to determine the specific conformation for the hexose carrier substrate there appeared to have been very little further work at relating substrate affinity with structure.

It was hoped that a full structure activity relationship would enable the shape of the carrier site to be determined and the groupings taking part in binding to be identified. Unfortunately only a limited number of glucose derivatives were available and as a consequence only very general conclusions can be drawn.

The lack of inhibitive action of the non-reducing sugars compared with the relatively high affinities of sugars bearing the same group attached at

another position e.g. trehalose and maltose, α-methyl glucoside and 3-O-methyl glucose, indicates that freedom of the C-1 position is a condition of binding.

The importance of the hydroxyl group at C-1 for hydrogen bonding was not studied since the '1-deoxy glucose' - 1,5-anhydroglucitol - was not available however, Evans et al. (1969) give a graph of the activity of this compound from which the half-saturation constant may be estimated as 45 mM at 37°C. This is a very great reduction in affinity compared to glucose and it has been suggested that the substrate is bound covalently to the carrier (Langdon & Sloan, 1967), but this has been disproved (Evans et al., ibid).

There has been a certain amount of debate about the ideal conformation of the C-1 position. The β-anomer fits best to the C1 conformation and this generally shows the greater activity, for example Sen(1960) found that freshly made solutions (β-glucose predominating) were more active than ones which had been standing (α-glucose predominating). This effect however must be very small since it was not observed in these experiments. Whether this difference in activity is due to a lower stability factor of the α-anomer or follows from steric hindrance is a matter for conjecture. It may perhaps be that hydrogen bonding to the C-1 hydroxyl group occurs at a point on the carrier site lying intermediate to the two alternative positions the group can adopt.

Whilst the hydroxyl group on C-1 is very important for binding the converse is true of that on the C-2 position, 2-deoxy glucose having a half-saturation constant about half that of glucose. In this case it would seem that glucose suffers steric hindrance and that reducing the hydroxyl group allows the sugar to bind more closely to the carrier site. Since the free energy change of dissociation is the same for glucose and 2-deoxy glucose it may be assumed that the oxygen of the C-2 group does not take part in this binding.

The high affinity of the 3-O-methyl and 4,6-O-ethylidene glucose derivatives indicate that these positions are not susceptible to steric hindrance from equatorially placed groups. The low affinities of maltose and cellobiose in which very bulky groups are attached equatorially suggests that there is nevertheless some steric effects but these may be the result of folding about the glycosidic linkage.

It would be interesting to discover the effect of reducing the hydroxyl groups on these atoms (C 3,4 or 6) to see whether they account for any hydrogen bonding but in a recent paper Barnett et al. (1973) have studied this problem in more detail and they have suggested that there is a hydrogen bond associated with the C-3 position and possibly one associated with C-4. There is also a hydrophobic region near the C-6 position, as a result of which the 6-deoxy, 6-fluoro derivative of glucose has the lowest half-saturation constant (1.8 mM at 36°C) for any sugar yet determined. The other findings of their study are in broad agreement with those outlined above.

A glucose derivative which has not been studied but which might be interesting is 5-thioglucose. In this the oxygen atom in the ring is replaced by a sulphur atom and the C-5 position therefore would not be susceptible to hydrogen bonding. Attempts to use two charged glucose derivatives was without success. Glucosamine (2-amino,2-deoxy glucose) and glucuronic acid (xylose-5-carboxylic acid) both produced excessive changes in surface charge with optical changes which masked the glucose exit.

The behaviour of the disaccharides is more difficult to interpret. Non-reducing sugars are without effect because of the blockage of the C-1 position but several reducing disaccharides are also inactive. Glucose-6-phosphate is prevented from binding presumably by the size or the charge of the phosphate group but lactose and melibiose which do not inhibit transfer differ from cellobiose and isomaltose respectively only in the configuration of the C-4 of the second ring. The reasons for this difference

between the glucosyl glucosides and the glucosyl galactosides is not clear since in both the pyranose rings take up the C1 conformation. However, cellobiose and isomaltose are in themselves anomalous. Cellobiose has a ΔE of about 17 kJ $mole^{-1}$ compared to 42 kJ $mole^{-1}$ for maltose and glucose and although this may be due to a different degree of binding it would seem more likely that a change in the equilibrium conformation of the sugar with temperature alters the amount of sugar available for inhibition. The loss of activity of isomaltose solutions with time is suggestive of mutarotation. A value of $+122^{o}$ is given as the specific rotation of isomaltose in water but there was no value available for a freshly made solution. The alternative possibility that the molecule is affected by membrane enzymes may be ruled out since hydrolysis would produce free glucose with a consequent increase in the degree of inhibition. Rotation about the glycosidic linkage may provide one explanation of these various anomalies but resolution of the problem must wait until the full conformation of each disaccharide is known.

The allosteric behaviour of tetrahydropyran suggests that this compound does not react with the binding site but rather with the lipid parts of the carrier. Preliminary experiments with 1-hydroxyvaleraldehyde which exists in solution partly as a pyran ring with a C-1 hydroxyl group indicate that this does not cause any inhibition, perhaps because of its lower lipid solubility. The inhibitive action of tetrahydropyran and other lipid soluble reagents e.g. Triton-X 100 (Hunter, 1965) would suggest that the carrier has a high lipid content and this is supported by the findings of Jung et al, (1973) that it is not susceptible to pronase digestion.

With the exception of cellobiose and tetrahydropyran, all of the glucose derivatives show similar slopes for the Arrhenius plots. The energy change is about 42 kJ $mole^{-1}$ and this could correspond to the breaking of between two and four hydrogen bonds. This is in agreement with the findings of Barnett et al. (1973) who from a study of structure activity

relationships suggested that hydrogen bonding occurs between the sugar and the C-1, C-3 and C-4 and that a further unidentified bond occurs at the C-6 position.

Because the derivatives show the same thermodynamic constants it may be concluded that they are all reacting with the same components and since maltose and ethylidene glucose are not transported by the carrier, the half-saturation constant determined by the Sen-Widdas technique must relate to a reaction at the outer surface of the membrane.

The mode of Penetration of Ethylidene Glucose.

Undoubtedly the most important finding of this study was the discovery of 4,6-O-ethylidene glucose as a carrier substrate. It appeared likely to react with the carrier site since it is a planar molecule with an intact reducing group and this assumption was borne out by its high affinity for the hexose transfer system. As has been shown in chapter 4. ethylidene glucose is not transported by the carrier despite its high infinity. Instead it is concluded from the study that ethylidene glucose enters the red cell by a process of diffusion. This ability to penetrate the cell membrane must be due to the hydrophobic nature of the molecule, resulting from the inclusion of the C-4 and C-6 oxygen atoms in other type linkages with the ethylidene group. This increases the partition coefficient by two orders of magnitude. It may be noted that methylation of the hydroxyl groups of glucose results in an increase in the partition coefficient of approximately one order of magnitude for each group methylated, thus α-methyl glucoside has a partition coefficient of about 10^{-5} and the observation that this compound penetrates the red cell very slowly (Bowyer, 1957) may be interpreted as due to a possible diffusive process. This could be checked using chlorpromazine which causes an increase in the rate of passive diffusion as has been shown with ethylidene glucose (Baker & Rogers, 1972). By decreasing the partition coefficient a further order of magnitude to that

of glucose the rate for diffusion becomes vanishingly small, or absent.

Since ethylidene glucose is bound by the carrier the cause of its inability to be translocated must be considered. According to Krupka (1972) the transfer of hexose results in a conformational change in the carrier which permits FDNB to bind and using 2-deoxy glucose and maltose, he has shown that the attachment of maltose to the outer surface of the membrane results in the protection of the carrier on both sides. This indicates that the effect of binding is transmitted through the membrane and as one explanation of this Krupka suggests that the carrier may consist of two components linked together. If FDNB binding takes place when the components face into the membrane then the results in chapter 4 would indicate that ethylidene glucose like maltose will not permit introversion to occur. Thus it would appear that the binding site is not exposed to a lipid environment during introversion otherwise ethylidene glucose might enter the membrane even if it could not be transferred between the components.

Almost certainly it is the size of the molecule which prevents it from being transported and it is hoped that the slightly smaller 4,6-O-methylene glucose which is in the course of preparation may yield more information about the limiting size for transfer. Rosenberg (1961) has indicated that 6-O-methyl glucose is capable of being transported but the rigidity of the dicyclic derivatives may be a factor in preventing introversion of the carrier components.

The effect of ethylidene glucose on isotopic fluxes.

The finding that ethylidene glucose is capable of inhibiting the hexose carrier although entering the red cell by simple diffusion suggested that it may be used as a competitive inhibitor at the inner surface of the membrane without affecting the saturation of the outer surface. Thus in contrast to methods depending on mathematical analysis the use of ethylidene glucose offered a direct means of determining any asymmetry in the hexose transfer

system.

Measurement of the inhibition of 20 mM glucose exchange by ethylidene glucose indicates that there is a difference in the affinities for the carrier at the two sides of the membrane, of the order of 6-7 times relative to glucose, the outside having the higher affinity. Repetition of this experiment at 10 mM glucose enabled the actual half-saturation constants to be determined for each surface and these were found to be 1.2 and 12 mM for glucose and 1.8 and 70 mM for ethylidene glucose at the outside and inside respectively.

This degree of asymmetry constitutes strong evidence against the simple carrier model and also against most of the models published. The only model in which asymmetry is a property of the system is that proposed by Geck (1971) which has been dismissed by Lieb & Stein (1972), on theoretical grounds. The asymmetry measured by ethylidene glucose inhibition must be a property of the whole system and not just one part of it. This excludes the tetramer of Lieb & Stein (ibid.) since this model requires a pair of asymmetric systems arranged contrariwise so that symmetrical inhibitory properties should be displayed.

The existence of different affinities suggests that the carrier sites at each surface of the membrane are distinct from one another and Professor Widdas has postulated a new model consisting of pairs of components with different affinities arranged in series across the membrane. The kinetics of such a two component model are discussed later but the concept is useful in analysing the other results.

The results from sorbose exchange fluxes confirm the observation of Sen & Widdas (1962a) that the half-saturation constant for glucose determined by glucose exit is less than that measured by sorbose entry. This discrepancy in the simple carrier therefore is not due to the use of simplifying assumptions but indicates a fundamental difference in the mechanism of transfer. Widdas (cited Bowyer, 1957) gives a value of 44 mM

for the half-saturation constant of maltose determined from sorbose inhibition and this is about $1\frac{1}{2}$ times the value determined in this study with glucose exit. Thus comparison of the transported and non-transported sugars shows that half-saturation constants of the former are increased some $2\frac{1}{2}$ times while the latter suffer an increase of only $1\frac{1}{2}$ times. This may mean that sorbose is capable of binding more readily with a complexed carrier site than with a free one, which may be due to the displacement of the occupying sugar or may be the result of some conformation change which occurs on binding and persists long enough after dissociation to enable the sorbose to bind more readily than normal. An additional effect must also be occurring during the transfer process to account for the larger change in the affinities of the transported sugars and this is discussed in the kinetic treatment of the model. The assumption of Levine et al. (1971) that the inhibition of sorbose fluxes provides a measure of the true dissociation of the carrier sugar complex now seems to be erroneous.

The exchange rate of sorbose ($0.32\ min^{-1}$) may be compared with the rate for sorbose entry ($0.34\ min^{-1}$) obtained in chapter 6. Unlike the rates for glucose transfer these two values are identical within limits. This is further evidence that sorbose transfer depends on the saturation of the components at the surface, since with low saturation the possibility of exchange between components becomes small and only the unidirectional fluxes are significant.

Effect of Chlorpromazine on Hexose Transfer.

Chlorpromazine produces a complex pattern of effects on hexose transfer which appear to result from its progressive uptake by the lipid parts of the carrier. Except at near-haemolytic concentration CPZ does not affect the affinity of the carrier (Baker & Rogers 1972). In terms of the two component model this has, in fact only been demonstrated for the outer component and a study of the variation of the affinity of the inner component might prove interesting. Nevertheless it appears that the actions of

chlorpromazine interfere with the rates at which sugar transfers take place. The first effect to be observed in this study was the apparent "doubling" of the sorbose transfer rate by high concentrations of CPZ. Sorbose transfer is limited by the degree of saturation of the carrier and therefore this increase in rate must by the result of an increase in the number of effective sites. This may suggest that the carrier has two sites at each surface one of which is not normally available for transfer.

The action of CPZ may be to uncover this second site or if the site is made inoperative by binding to the first site, CPZ may interfere with the conformational changes involved. An interesting possibility put forward by Professor Widdas is that two pairs of components are combined together to form a negatively co-operative linked tetramer. This scheme was originally proposed to explain exchanges between saturated components without postulating a pool of free sugar in the membrane but the transport rate of this system could be doubled if CPZ were capable of separating the components into two parallel dimers.

The acceleration of glucose exits by CPZ shown by Baker & Rogers (1972) would also seem to be related to this phenomenon but since a four-fold increase in rate is apparently involved here, some change in the net transfer velocity must also occur. The second phase of the inhibition of glucose exit does not give the same intercepts on the graph as the first phase and this appears to be due to the combined effects of inhibition and acceleration.

Another unusual observation of this study is that CPZ will inhibit net transfer about two-fold whilst leaving the exchange fluxes unaffected. This would appear to indicate that there are two different processes involved in transfer, one for exchange and one for net transport. In terms of current models this seems unlikely and an alternative explanation might be considered, for example CPZ may be unable to react with the carrier when both components are saturated. This observation is the reverse of an effect

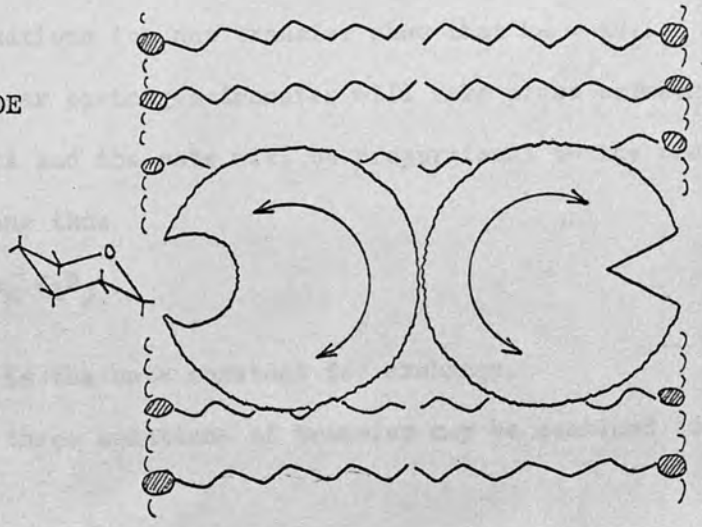
described by Zipper & Mawe (1972) where cells were treated with Phospholipase C and an inhibition of exchange was found without a corresponding inhibition of maximal net transfer. Since both reagents act on lipids the above findings suggest that some of the rate-controlling processes occur in a lipid region of the carrier system.

A New Model.

[The equations in this section were derived by Prof. Widdas]

It was the intention at the beginning of this study to obtain information which would enable a new carrier model to be formed. The difference in affinity of the carrier for its substrates that has been found between the inside and outside of the membrane provides just such information and from this finding a new model has been postulated by Professor Widdas.

The difference in affinities suggest that more than one binding site may be involved in transfer and this leads to the assumption that there are several components arranged in series across the membrane, each possessing a different affinity for the substrate. Transport occurs by a sugar passing from one component to the next and exchange is possible by the simultaneous transfer of sugar between two adjacent components. The simplest form of this model to consider is that consisting of just two components, one at the inner face of the membrane the other at the outer surface.



The kinetics of such a system may be derived from mechanisms developed earlier (Bowyer & Widdas, 1958) to explain transfer along a row of sites in a pore. In this model net transfer will occur when sugar passes from a saturated component on one side of the membrane to an unsaturated component on the other and will proceed at a rate proportional to the product of the saturation of the first component and the unsaturation of the second component. This may be represented by the equation,

$$\frac{ds}{dt} = k_1\theta_1 (1-\theta_2).$$

where k_1 is the rate constant for transfer from side 1 to side 2 and θ is the fraction of components saturated on each side respectively. Since the system is asymmetrical θ_1 will not equal θ_2 if the concentrations are the same on each side of the membrane and in order not to contravene the second law of thermodynamics the rate constants for the net transfer of sugar in each direction must be different. Hence

$$\frac{ds}{dt} = k_2\theta_2(1-\theta_1).$$

will be the rate of transport from side 2 to side 1.

If the saturation of component is taken as $C/(C+\varphi)$ by analogy with Langmuir's adsorbsion isotherm, where C is the concentration in the medium and φ is the half-saturation constant. The other component with a different affinity will have a saturation given by $C/(C+a\varphi)$, where 'a' is the asymmetry factor. Substituting $C/(C+\varphi)$ for θ_1 and $C/(C+a\varphi)$ for θ_2 the above equations for net transfer show that $k_2 = ak_1$.

For sugar exchanges transfer will take place between two saturated components and the rate will be proportional to the product of the two saturations thus

$$\frac{ds}{dt} = k_x\,\theta_1\theta_2$$

where k_x is the rate constant for exchange.

These three equations of transfer may be combined to give a complete

equation for the transport of a single sugar:-

$$\frac{ds}{dt} = \frac{ak_1\varphi(C-S) + k_x(CS-SC)}{(C+\varphi)(S+a\varphi)}$$

where C is the concentration on the outside of the cell and S, since isotonic volumes are maintained, is the concentration on the inside. The placing of φ and $a\varphi$ is purely arbitrary but with 'a' greater than unity the term '$a\varphi$' relates to the inside of the cell according to our results.

This model provides an explanation of many of the discrepancies found in the simple carrier model while still showing the basic kinetics of transport demonstrated by Widdas in 1954.

For net flux the equation becomes

$$\frac{ds}{dt} = \frac{ak_1\varphi(C-S)}{(C+\varphi)(S+a\varphi)}$$

If $\varphi \gg C$ and $a\varphi \gg S$

$$\frac{ds}{dt} = \frac{k_1(C-S)}{\varphi}$$ ie: diffusion type equation

If $C \gg \varphi$ and $S \gg a\varphi$

$$\frac{ds}{dt} = ak_1\varphi\left(\frac{1}{S}-\frac{1}{C}\right)$$ ie: near-saturation type equation

Since 'a' has a value of about 10 the latter equation will be rather approximate unless S is about a hundred times greater that φ.

For the Sen-Widdas procedure where S is made much greater than $a\varphi$ and C.

$$\frac{ds}{dt} = \frac{-ak_1\varphi}{(C+\varphi)}$$

giving a maximal rate of ak_1 when C=0 and a half-maximal rate when $C=\varphi$. Thus the Sen-Widdas plot gives values for ak_1 and the outside half-saturation constant φ and this latter is in agreement with the conclusion from the Arrhenius plots of maltose and other glucose derivatives.

The zero-trans procedure of Karlish et al (1972) puts C equal to zero and varies S thus the equation becomes

$$\frac{ds}{dt} = \frac{-ak_1S}{(S+a\varphi)}$$

This will also give ak_1 for the maximal transfer rate but a plot of

1/S versus 1/rate will give a half-saturation constant of $a\varphi$. Thus this method determines the value of the inner half-saturation constant.

The exchange flux is determined by making C = S thus the equation for the uni-directional flux becomes

$$\frac{ds}{dt} = \frac{ak_1\varphi C + k_x C^2}{(C+\varphi)(C+a\varphi)}$$

if C is an order of magnitude greater than φ and k_x greater than ak_1 than the equation approximates to

$$\frac{ds}{dt} = \frac{k_x C}{(C+a\varphi)}$$

from which a Lineweaver-Burk plot will give a maximal transfer rate of k_x and a half-saturation constant of $a\varphi$. Thus this model explains as to why the zero-trans and exchange procedures give similar values for the half-saturation constant but different values for the maximal transfer rate while the reverse relationship holds between the zero-trans and glucose exit procedures.

One transformation which does not agree with the observed results is that which refers to the zero-trans entry procedure where S=0 and C is varied. Thus the equation simplifies to

$$\frac{ds}{dt} = \frac{ak_1\varphi C}{(C+\varphi)a\varphi} = \frac{k_1 C}{(C+\varphi)}$$

This is a suspect method in intact red cells as pointed out elsewhere but in a recent study Taverna & Langdon (1973) have attempted to measure sugaruptake by ghosts preloaded with glucose oxidase. They have obtained values of 56 mM (litre packed cells)$^{-1}$min.$^{-1}$ and 11 mM for the maximal rate and the half-saturation constant respectively at 15.5°. These values are in good agreement with the values obtained in this study for ak_1 and $a\varphi$ not k_1 and φ as the equation predicts. The reason for this is not clear and further results of this type would be useful. It is possible that there is some alteration in the properties of the ghosts, perhaps, even an inversion of the membrane, during the resealing procedure.

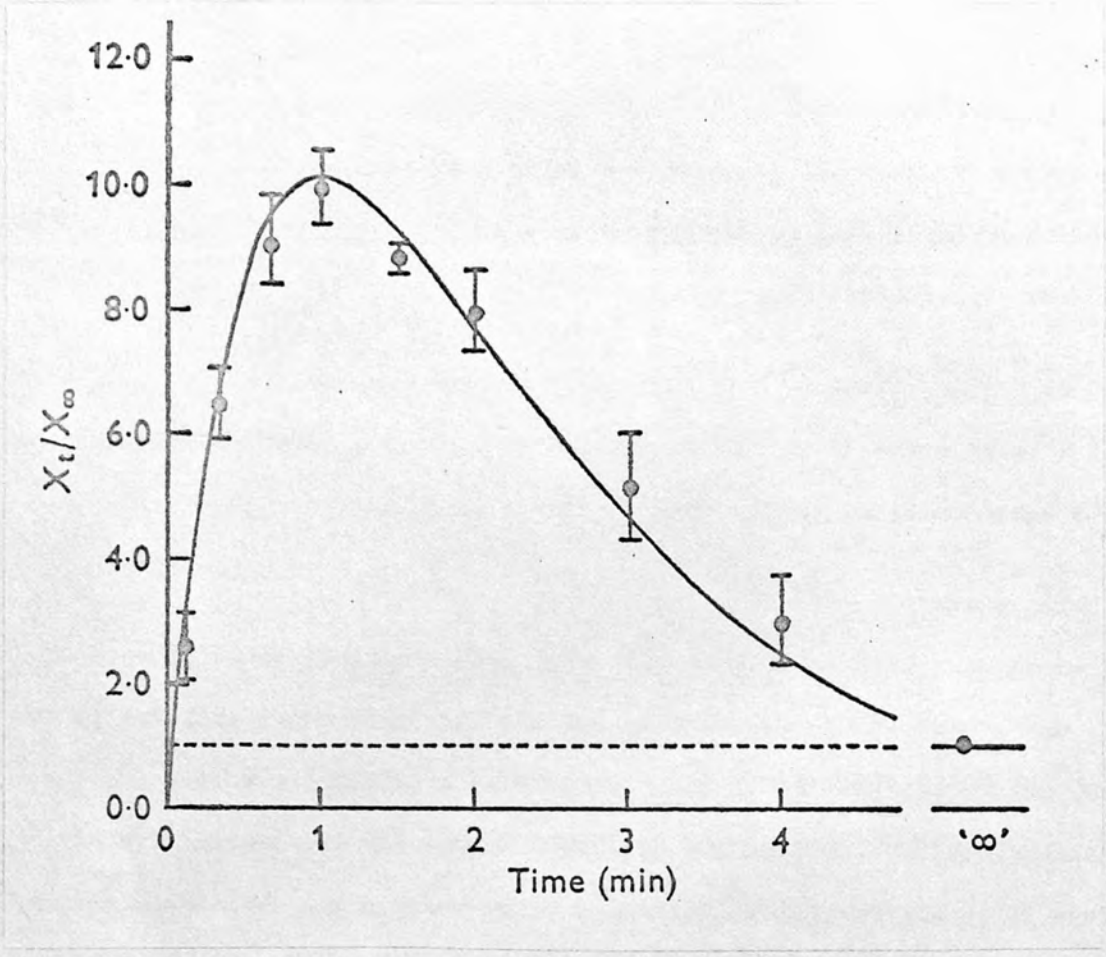


Figure 25.

Uphill transfer of 4 mM ^{14}C- glucose induced by the counterflow of 76 mM unlabelled glucose at 16°C. The amount of radio-activity inside the cells is expressed relative to the amount at equilibrium. Points are mean of 5 determinations and the continuous line has been calculated from the two component system described in the text.

The model does however, explain the higher value for the half-saturation constant as measured by sorbose inhibition. The main equation may be expanded to include a second sugar. Thus for the influx of sugar 'A' in the presence of sugar 'B'

$$\frac{ds_a}{dt} = \frac{ak_a\varphi_a C_a + k_x S_a C_a + k_e S_b C_a a\varphi_a/b\varphi_b}{(C_a + \varphi_a(1+C_b/\varphi_b))(S_a + a\varphi_a(1+S_b/b\varphi_b))}$$

where k_a corresponds to k_1 and k_e is the rate constant for the exchange of sugar 'A' with sugar 'B' and 'a' and 'b' are the subscripts for the two sugars. If 'A' represents sorbose and 'B' the inhibiting sugar then under the conditions of our experiements. $\varphi_a \gg C_a$ and S_a and $C_b = S_b$ allowing the above equation to be simplified to

$$\frac{ds_a}{dt} = \frac{ak_a\varphi_a C_a + k_x S_a C_a + k_e C_a C_b a\varphi_a/b\varphi_b}{\varphi_a(1+C_b/\varphi_b) \cdot a\varphi_a(1+C_b/b\varphi_b)}$$

Thus when $C_b = \varphi_b$, ds/dt is not half-maximal but greater by a factor of approximately $(b+k_e/k_a)/(b+1)$. Owing to the approximations involved, the direct determination of the individual parameters for transfer is not possible in this case but with more information an indirect estimate could be obtained.

The two-sugar equation may also be used to predict the time course of counter transport fluxes. This is seen in Fig. 25, where the calculated values for an uphill transfer of glucose-^{14}C are compared with the experimental results. If both sugars are glucose then $a\varphi_a = b\varphi_b$ and $k_e = k_x$ so that putting $C^* = C_a$ = labelled glucose and $C = C_b$ = cold glucose, the equation for influx becomes

$$\frac{ds^*}{dt} = \frac{ak_a\varphi C^* + k_x S^* C^* + k_x C^* S}{(C^* + \varphi(1+\frac{C}{\varphi}))(S^* + a\varphi(1+\frac{S}{a\varphi}))}$$

At first $S^*=0$ and S is large so that it is the exchange of C^* with S that is responsible for the accumulation of radioactivity in the cell. The maximum accumulation is reached when $ds^*/dt = 0$ and by substituting values for the various parameters in the appropriate equations it can be shown that at this point the value of S is fairly low thus the flux which follows

the peak is due almost entirely to the loss of the accumulated sugar. If counter-transport is induced between different sugars as has been done by Miller (1965), the simplifications used above are not applicable and a full knowledge of the transfer parameters k_e and $b\varphi_b$ is required to permit calculation of the fluxes.

Future Work.

The present position is similar to that just over ten years ago when Sen & Widdas (1962a) developed the glucose exit technique to measure the maximal transfer rate and the half-saturation constant 'φ' of hexose transport. It is now apparent that these parameters do not completely describe the system, two additional parameters, the maximal exchange rate and the asymmetry factor also being required. These new parameters may be determined by the inhibition of exchange transfer using ethylidene glucose as described in this thesis. Radio-isotope experiments, however, have neither the convenience nor the rapidity of the Sen-Widdas technique and some consideration must be given to deciding the most profitable way of using them to obtain further information about hexose transfer mechanisms.

The outstanding problem at present is to find the way in which transfer within the membrane occurs. The model presented here acknowledges the difference between the maximal exchange and net transfer rates but gives no indication of the underlying cause of this difference. Further there is the variation in the rates of transfer of different sugars which the model also allows for, but which has not yet been explained. Studies involving the use of inhibitors such as chlorpromazine which can distinguish between the two types of transfer may give some indication of the processes of translocation but to understand the manner in which the sugar molecules pass between components requires a different means of analysis. This would involve a study of the variation of transfer parameters with sugar structure so that interaction of the sugars with the binding sites inside and outside

the membrane could be determined. An interesting consideration arises from a table of data published by Lieb & Stein (1972) part of which is shown below, together with the results of this study.

TABLE IV.

Hexose Transfer Parameters

Sugar	Temp.	Half-saturation Constant (mMolar)				Maximal Transfer Rate (m.mole $l^{-1}min^{-1}$)		
		S-W	Exch.	Z-T	'a'	Exch.	Net Flux	ratio
Glucose	16°	1.2	12	-	10	190	70	2.7
Glucose	20°	1.8	32	25	16*	360	140	2.6
Mannose	20°	7	72	-	10	420	-	-
Galactose	20°	12	147	165	13*	460	220	2.1
Glucose	37°	4	-	57	14	-	1060	-

The asymmetry factor 'a' has been calculated by dividing the half-saturation constants for exchange and zero-trans by those for Sen-Widdas determination, figures marked '*' are the mean of the two possible values.

It will be noted that both the asymmetry factor and the ratio of the flux rates are similar for each sugar and this suggests that the underlying mechanism is the same in each case. It would seem to be permissible therefore to look at the empirical relationship which can be obtained by plotting the half-saturation constant against the exchange rate for each sugar since the other parameters will differ from these by a common factor. Such a plot of the data for the three sugars measured at 20°C is found to be hyperbolic and can be represented by

$$k = \frac{T\,\varphi}{\varphi+\mu}$$

where k is the maximal rate of exchange for the sugar concerned, φ its half-saturation constant for exchange, T is the asymptotic value and μ is

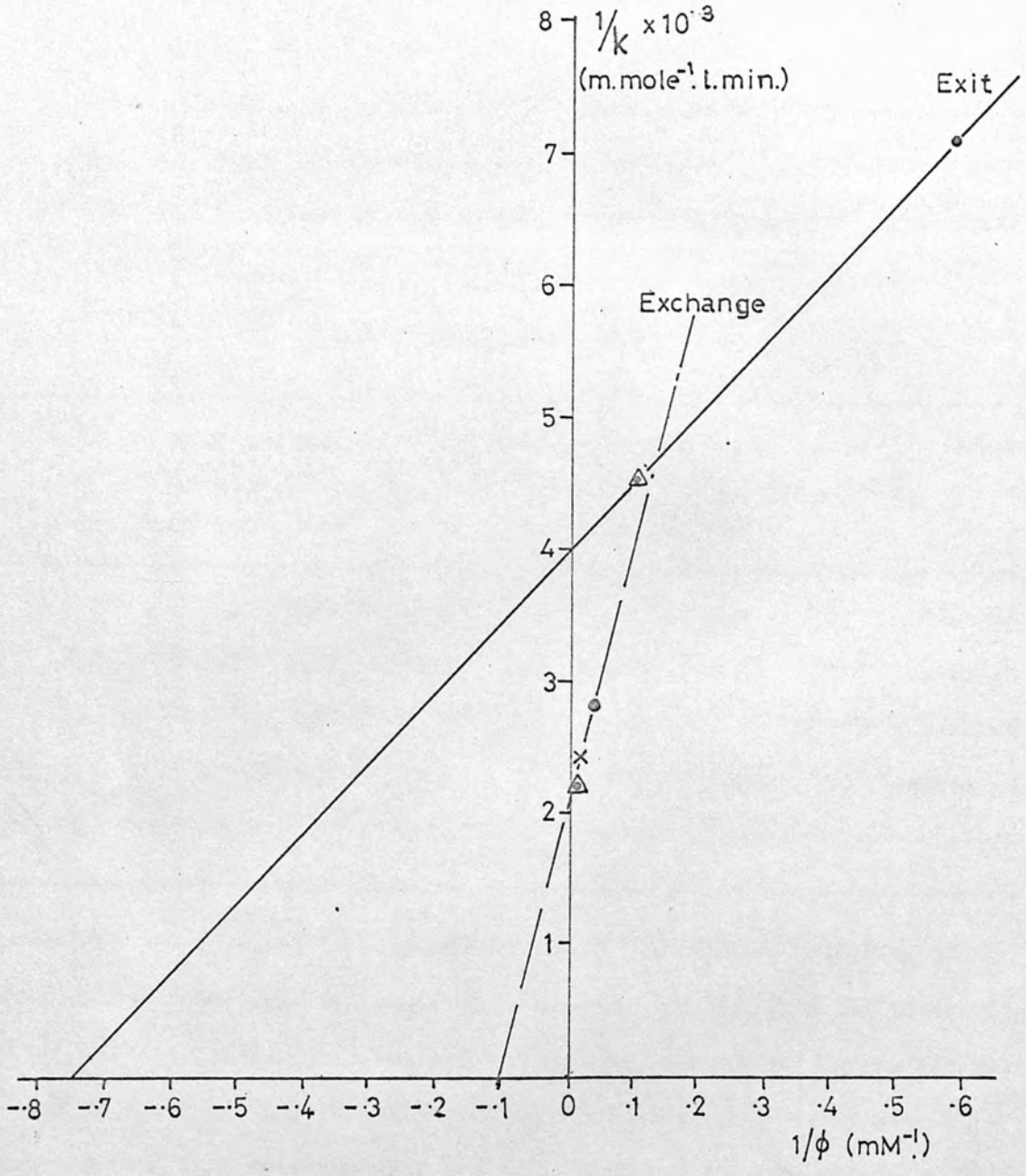


Figure 26.

Double reciprocal plots of the maximal transfer rate versus half-saturation constant for net and exchange transfer of glucose (●), mannose (×) and galatose (▲) at 20°C. Data from Lieb & Stein (1972).

the value of φ at which $k = \frac{1}{2}T$. A double reciprocal plot of the data is seen in Fig.26, which includes both exchange and net transfer parameters.

The theoretical consequences of this empirical relationship are relevant to the consideration of the transfer mechanism. If the affinity of the carrier for a sugar is very high i.e. the half-saturation constant is close to zero, then the sugar will associate readily with the carrier but will have difficulty in dissociating. The rate of transfer will therefore be very low for a given degree of saturation. Conversely if the half-saturation constant is very high i.e. the affinity approaches zero, the sugar will leave the carrier very readily so that assuming the same degree of saturation could be obtained, transfer would be rapid. However, in the extreme case the rate of transfer would be limited by the rate of movement of the carrier components and the reciprocal of this rate corresponding to 1/T will be given by the intercept on the ordinate of the above graph. This has a value of approximately 500 m.mole $l.^{-1} min.^{-1}$ for hexose exchange and might be regarded as a parameter of the membrane translocation process.

The intercept on the abscissa of this graph $(1/\mu)$ is more difficult to interpret since it corresponds to the affinity of the sugar which is transferred at half the maximal exchange transfer rate. It appears to represent the optimum value for the affinity since for a given concentration the rate of movement of such a sugar will be equal to or greater than that for a sugar with a different affinity. According to the above graph the reciprocal of this parameter has a value of about 10 mM at 20° and is close to the half-saturation constant that would be expected for 2-deoxy glucose, at this temperature. It would be interesting to find out whether this parameter shifted closer to the half-saturation constant for glucose as the temperature was raised to 37°C since the system would then have its optimum rate of transfer at the concentration at which the intended substrate was presented to it.

This hypothesis is largely conjectural since only three points are available for the graph but Lieb & Stein (1970) use a similar concept for calculating the probabilities of internal transfer in their tetramer model, and further studies with other labelled sugars aimed at verifying this idea, may give additional information on the transfer process for example if a sugar deviates from the above relationship perhaps due to steric effects, it may permit some of the geometry of transfer to be inferred.

The fairly consistent values for the asymmetry factors of glucose and the other sugars irrespective of the half-saturation constants suggest that the binding site of the inner surface is similar to that at the outer surface since binding to some other part of the pyranose ring would presumably alter the relative affinities. Examination of the results of Barnett et al. (1973) shows that a factor of ten is introduced if one of the hydrogen bonding groups is removed. Thus glucose:1,5-anhydro glucitol, glucose:3-deoxy glucose and 2-deoxy glucose:1,2-dideoxy glucose all show half-saturation constant ratios of about ten. The inner component therefore may lack the hydrogen bonding potential at C-1 or C-3 position whilst the very large asymmetry factor of ethylidene glucose suggests some source of steric hindrance near the C-6 or the C-4 positions.

If this is the case then it would be possible to find a substance perhaps, 1,5-anhydroglucitol which has the same affinity at both surfaces and this would require that $k_1 = k_2$ so that it must be possible to withdraw the asymmetry characteristic of the system. If such a substance could be investigated then it may give further information about the way transport occurs.

Kinetic characterisation will not in itself provide a full explanation of the phenomenon of sugar transfer and further work must be directed at identifying the structure of the membrane components responsible. The carrier appears to be an elusive molecule since despite many attempts at

its isolation none have proved successful. In part this may be due to the nature of the carrier for although it shows many of the properties of proteins it is susceptible to lipophyllic reagents. The other cause is the labile nature of the bond that it forms with its substrates. In the isolated state, binding will not be recognised by the transfer that normally follows in the intact system and therefore some reagent less specific than the substrate must be used to label the isolated carrier. This has been attempted by Eady & Widdas (1973) using the differential uptake of radio-active FDNB in the presence of 2-deoxy glucose and ethylidene glucose but the carrier could not be detected because of the high level of non-specific binding of the label. If this could be reduced, perhaps by preincubating in ethylidene glucose and unlabelled FDNB, then this technique seems among the best methods of identifying the carrier components that are available.

Ultimately it may depend upon the techniques of molecular biology to provide the explanation of how the carrier molecules are capable of transporting their burdens across the hydrophobic barrier of the membrane.

SUMMARY.

I. A review has been made of current ideas on the mechanism of hexose transfer and the effect of certain inhibitors upon it.

II, A study was made of the apparent half-saturation constants of a number of sugars and their variation with temperature.

III. A glucose derivative, 4,6-O-ethylidene glucose was studied and found to enter the red cell by simple diffusion although reacting with the hexose carrier.

IV. The unique properties of this compound were used to investigate the degree of asymmetry of the hexose system to inhibition at the two surfaces of the membrane.

V. The value obtained by Widdas (1954) for the inhibition constant of glucose using sorbose transfer was confirmed as being larger than the value determined by Sen & Widdas (1962a) and the work was extended using a radio-active technique.

VI. The various effects of Chlorpromazine on different sugar fluxes were investigated in an attempt to analyse its mode of action.

VII. Two new criteria for models of hexose transfer have been suggested by this work, (i), the carrier is asymmetrical, the inside having a lower affinity than the outside. (ii), the net flux has a different basis from the exchange flux.

VIII. A new model which takes account of these criteria has been discussed and some points for future research have been suggested.

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A glucose derivative which enters the human red cell by simple diffusion

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Ethylidene glucose (4-6-*O*-ethylidene α-D-glucopyranoside) competitively inhibits glucose exit from human erythrocytes with a half-saturation constant of about 5 mM at 36° C. This is similar to 3-*O*-methyl-glucose, also with a half-saturation constant of 5 mM at 36°, and is comparable to the half-saturation constant of glucose (4 mM) measured in the same way (Sen & Widdas, 1962).

In penetrating human red cells, however, ethylidene glucose does not show saturation or decrease of the apparent permeability coefficient at high concentrations as seen with both glucose and 3-*O*-methyl-glucose.

Ethylidene glucose does not appear to use the hexose system for the following reasons:

(1) Glucose at 38 mM produces no slowing of ethylidene glucose penetration as it does with sorbose penetration (Widdas, 1954).

(2) Phloretin (10^{-5} M) has no effect.

(3) Incubation of cells with fluorodinitrobenzene (FDNB) (Bowyer & Widdas, 1958) to produce up to 95 % inhibition of glucose exit is without effect on ethylidene glucose penetration.

(4) Ethylidene glucose penetrates red blood cells from adult guinea-pigs (which do not show glucose penetration by optical methods) at rates comparable but slightly faster than for human red cells.

The absence of a saturation effect has been confirmed by measuring volume changes during the entry of ethylidene glucose with trans-membrane gradients of 90–60 mM at progressively increasing concentrations up to 360 mM. Osmotic haemolysis of human and adult guinea-pig red cells also occurs in isosmotic solutions of ethylidene glucose (buffered to pH 7·4 with 0·03 M phosphate) taking 3–3·5 min for 50 % haemolysis at 36° C. The time course of haemolysis is unaffected by 0·3 M glycerol or 10^{-4} M-Cu^{2+}. Another anomaly relative to glucose and 3-*O*-methyl-glucose is that the inhibitory reaction with FDNB proceeds more slowly in the presence of 76 mM ethylidene glucose than in a saline control whereas glucose and 3-*O*-methyl-glucose potentiate the FDNB reaction.

The exit of sugar from cells equilibrated with 76 mM glucose or 3-*O*-methyl-glucose in a suspension medium containing 4 mM ^{14}C-labelled glucose induces an uphill transfer of labelled sugar which reaches *ca.* 24 mM inside the cells within 15 sec. No such uphill transfer is seen with cells equilibrated with ethylidene glucose.

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It would appear from the evidence available that, while reacting strongly with ethylidene glucose, the hexose transfer system is incapable of demonstrably effecting its translocation.

It is postulated that the ethylidene grouping in ethylidene glucose lowers its hydrophilic character relative to the parent molecule so that although larger than glucose it is sufficiently lipid soluble to penetrate the cell by a process of activated diffusion through parts of the membrane different from those occupied by the hexose transfer system.

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The asymmetry of the hexose transfer system in human red cells towards ethylidene glucose

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Although 4,6-0-ethylidene-α-D-glucopyranose (ethylidene glucose) is a potent competitive inhibitor of glucose exit from human erythrocytes it is not translocated by the facilitated transfer system for hexoses but appears to enter red cells by simple diffusion (Baker & Widdas, 1972). Due to the slow rate of passage across the cell membrane the competition which ethylidene glucose exerts can be maintained predominantly at either side of the membrane for the short time (*ca.* 60 sec) which is needed to measure glucose exchange flux using a [^{14}C]glucose technique based on that described by Miller (1968).

In the present experiments volume changes were minimized by balancing the osmotic effects of the ethylidene glucose with malonamide intracellularly when ethylidene glucose was extracellular and with a mixture of malonamide and inositol extracellularly when ethylidene glucose was inside.

With glucose-glucose exchange measured at 20 mM and 16° it was found that the concentration of ethylidene glucose which reduced the exchange flux to half was $\bar{c}$ 200 mM when ethylidene glucose was inside the cells in contrast to 25–30 mM when it was on the outside.

Since ethylidene glucose exit from the cells does not induce an uphill transfer by counterflow (Baker & Widdas, 1972) and since the cell volumes were approximately constant the glucose exchange flux (J) was treated as one of simple competition to give:

$$\frac{1}{J} = \frac{1}{K}\left\{1+\frac{\phi_G}{[C]}+\frac{\phi_G}{\phi_I}\frac{[I]}{[C]}\right\},$$

where K is a constant, $[C]$ and $[I]$ are the concentrations of glucose and inhibitor respectively and ϕ_G and ϕ_I their half-saturation constants.

The slope of a plot of $1/J$ against $[I]/[C]$ which should represent $(1/K)(\phi_G/\phi_I)$, is more than six times greater when ethylidene glucose is on the outside than when it is on the inside. At the high concentrations of ethylidene glucose used intracellularly there is some effect due to a small glucose impurity in the reagent but correction for this would tend to increase the ratio.

Such a high ratio between the relative affinities of glucose and ethylidene glucose on the two sides of the membrane is inconsistent with a symmetrical system with similar chemical groupings exposed on the two sides. It is also unlikely that the components involved could show these different

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affinities at the two sides of the membrane (e.g. Geck, 1971) from simple causes such as a change in the ionic environment. Although conformational changes cannot be ruled out the possibility of different subcomponents on the outer and inner face of the membrane being involved in the hexose transfer system must be considered. If present such subunits are unlikely to be symmetrically arranged as proposed by Lieb & Stein (1970).

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EFFECTS OF PSYCHOTROPIC DRUGS ON THE ERYTHROCYTE PERMEABILITY TO GLUCOSE AND ETHYLIDENE GLUCOSE

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Abstract—The effect of chlorpromazine (CPZ) and a number of CNS depressant drugs on glucose penetration through human erythrocyte membrane has been investigated by an optical technique.

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EFFECTS OF PSYCHOTROPIC DRUGS ON THE ERYTHROCYTE PERMEABILITY TO GLUCOSE AND ETHYLIDENE GLUCOSE

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Abstract—The effect of chlorpromazine (CPZ) and a number of CNS depressant drugs on glucose penetration through human erythrocyte membrane has been investigated by an optical technique.

CPZ accelerated glucose exit at concentrations between 1×10^{-5} and 2×10^{-5} M at 36° but at higher concentrations inhibited transfer. This inhibition was rapidly and completely reversible. CPZ, trifluoperazine, prochlorperazine, promazine and promethazine were found to inhibit glucose exit approximately in the order of their chemotherapeutic potency. Imipramine also showed this effect but nealbarbitone, thiopentone and haloperidol did not.

CPZ affects the entry of glucose into erythrocytes in a biphasic manner similar to its effect on exit but at all concentrations it accelerates the penetration of ethylidene glucose which enters by diffusion.

CPZ had no effect on the inhibition of glucose transfer produced by incubation with dinitrofluorobenzene (DNFB) or on the enhancement of this inhibition by incubation in the presence of glucose and 2-deoxyglucose.

It is suggested that at low concentrations CPZ accelerates the movement of the glucose carrier within the membrane by effects on its charge environment. At higher concentrations interaction with the protein of both membrane and carrier presumably causes interference with carrier movement until at high concentrations haemolysis occurs. The relevance of these effects to the pharmacological action of CPZ is discussed.

THE EXISTENCE of facilitated diffusion of glucose from blood to brain[1–3] and blood to C.S.F.[4] has been established by experiments *in vivo* and a similar carrier mediated transport of sugars has been shown in brain slices.[5] The passage of glucose across the neuronal membrane may exert an important control over brain metabolism.[6] Alterations in glucose penetration have been postulated to explain both chlorpromazine induced hyperglycaemia[7] and the increased brain glucose concentration during anaesthesia and chlorpromazine treatment.[8] At present it is not possible to investigate the kinetics of glucose transfer and its modification by drugs in cerebral tissues using direct methods. The general properties of the system with respect to saturability, stereospecificity, affinity for various sugars and lack of effect of insulin and metabolic inhibitors appear to be similar to those found in the erythrocyte. The recent estimation by Bachelard[5] of a half-saturation constant, K_m, for glucose of the order of 5 mM in brain slices is comparable with that found for the erythrocyte.[9] In view of these findings and the amount of information available on both the membrane stabilizing effects of psychotropic drugs and on the kinetics of glucose penetration into the red cell, it was considered useful to investigate the effect of some psychotropic drugs, in particular chlorpromazine (CPZ), on the glucose transfer system of the erythrocyte.

METHODS

Blood was collected by venepuncture, heparinized and the cells washed three times in buffered saline before use. Blood was used up to 72 hr after collection without effect on the congruence of the results. The exit and entry of glucose and the entry of 4,6-0-ethylidene–D-glucopyranose (Koch–Light), "ethylidene glucose", were followed in an Ørskov-type photoelectric apparatus and the results analysed as detailed in Widdas[10] and Sen *et al.*[9] 2,4-Dinitro-l-fluorobenzene (B.D.H.)—"DNFB", was prepared and used as detailed by Bowyer *et al.*[11] Chlorpromazine HCl (May and Baker), nealbarbitone (May and Baker), thiopentone sodium (May and Baker), trifluoperazine HCl (Smith, Kline and French), imipramine HCl (Biorex), promazine HCl (Wyeth) and haloperidol (Roche) were studied. Drug solutions were prepared fresh before each experiment in a medium buffered at pH 7·2 containing Na^+ 155·8 mmole/l; K^+ 5·6 mmole/l; Ca^{2+} 4·3 mmole/l; Cl^- 163·9 mmole/l and HCO_3^- 1·8 mmole/l and these solutions were used as the suspending medium during both exit and entry experiments. In trial experiments it was found that preincubation in drug solutions gave results identical with those in which the preincubation had been in saline medium, the latter method was therefore more usually used. CPZ *per se* was found not to alter red cell volume under the conditions of these experiments.

RESULTS

Effects on glucose permeability. The effect of various concentrations of CPZ on the exit of glucose from erythrocytes previously loaded with 76 mM glucose was investigated. The results of experiments conducted at 36° are shown in Fig. 1 in which exit time is plotted against CPZ concentration. Since under these experimental conditions exit time is inversely related to the transfer rate such a plot is a modification of the Dixon plot used in enzymology and should be a straight line if inhibition follows Michaelis–Menten kinetics. As can be seen this is not the case: after an initial fluctua-

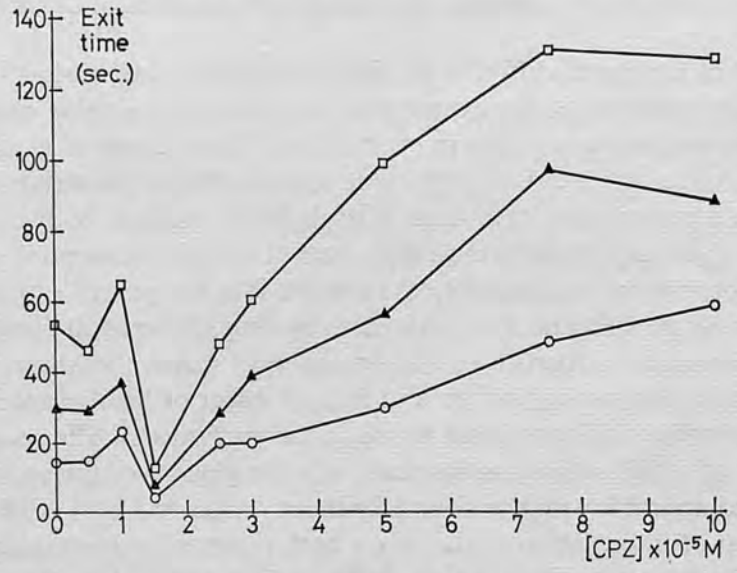

FIG. 1. The effect of chlorpromazine on the exit time of 76 mM glucose from human erythrocytes. The points represent the mean of between 3–12 determinations. External glucose concentrations: ○, 0·7 mM (Control); ▲, 4·5 mM; □, 10·1 mM.

tion the exit time drops sharply to about a third of its control value before rising in a non-linear manner and levelling off at around three times control value.

The same response was observed at 27°, 22°, and 17°, but with lower temperature the point of inflexion was found to occur at a higher concentration, e.g. it is 3×10^{-5} M at 22°, and the biphasic shape of the curve was emphasized. It is interesting that the CPZ concentration at which saturation of membrane stabilizing sites occurs was found to be approximately 2×10^{-5} M at 22° by Kwant and Seeman.[12]

Glucose entry at 36° showed similar changes in rate to those seen with the exits at that temperature, the point of inflexion lying between 1×10^{-5} and $2{\cdot}5 \times 10^{-5}$ M CPZ.

The influence of glucose in the external medium on exit at 36° is shown in Fig. 2 (Sen–Widdas plot). In such plots the intercept on the abscissa is equal to $-K_m$, the half saturation constant, and that on the ordinate is $1/V_{max}$, the reciprocal of maximum velocity. This also reflects the anomalous effect of CPZ on the velocity of glucose

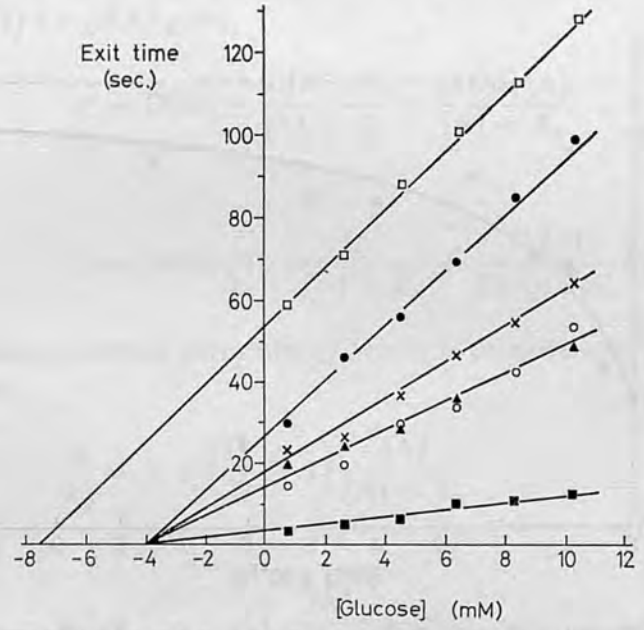

FIG. 2. Sen–Widdas plot of the exit times of 76 mM glucose from human erythrocytes into glucose solution containing chlorpromazine. Chlorpromazine concentrations: ○, Control; ×, $1{\cdot}0 \times 10^{-5}$ M; ■, $1{\cdot}5 \times 10^{-5}$ M; ▲, $2{\cdot}5 \times 10^{-5}$ M, ●, 5×10^{-5} M; □, $1{\cdot}0 \times 10^{-4}$ M.

transfer but shows that over the lower range of concentrations the half-saturation constant is unaffected. This indicates that the effect of the drug is non-competitive and that it is acting elsewhere than at the site of glucose binding. The alteration of the half-saturation constant by 1×10^{-4} M CPZ suggests that the binding site has become affected by the CPZ at this high concentration but this may be part of a progressive membrane involvement which leads to haemolysis at concentrations of this order.

These drug effects on the exit and entry of glucose were found to be completely and rapidly reversible upon washing the cells at all sub-lytic concentrations of CPZ.

The results of experiments with 1·4 mM DNFB support the findings that CPZ (10^{-5} to 10^{-4} M) does not interfere with the glucose binding site. CPZ exerts no influence on the irreversible inhibition of glucose transfer produced by DNFB nor does

it affect the enhancement of the development of inhibition by incubation with DNFB in the presence of glucose or 2-deoxyglucose. This latter effect has been interpreted as the stabilization by transported sugars of a conformation of the carrier which is favourable to DNFB combination and this conformational state of the carrier with its bound glucose is postulated to be an intermediate in the transport process.[13] This behaviour is different from that of some other substances: mainly detergents such as Triton X-100 and sodium lauryl sulphate, which have membrane stabilizing properties[14] and inhibit glucose penetration in a non-competitive manner but which also accelerate the development of DNFB inhibition.[15]

Effects on ethylidene glucose permeability. Because of the complex nature of the CPZ action on glucose permeability it was decided to study its effects on the permeability of a substance which penetrates the red cell by diffusion. Ethylidene glucose is a glucose derivative which appears to penetrate in such a manner[16] and its slow rate of penetration makes it suitable for study by the Ørskov technique. Fig. 3 shows the effect of CPZ

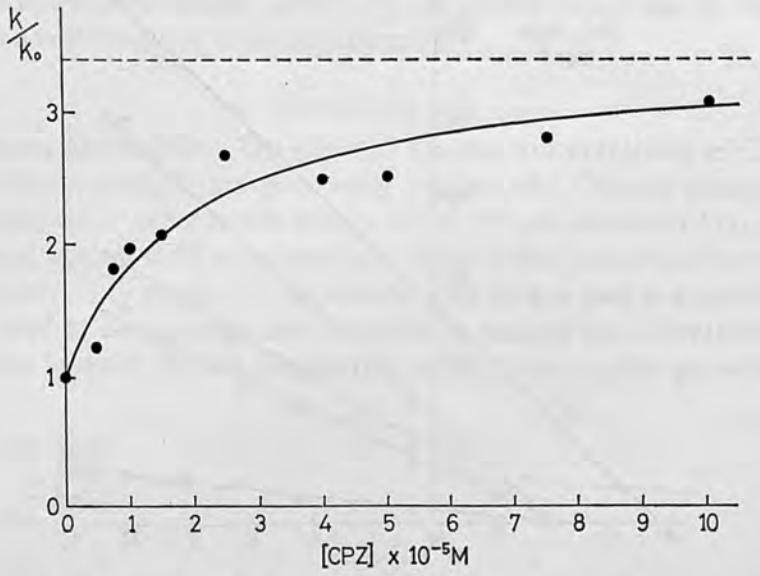

FIG. 3. The rate of diffusion of 38 mM ethylidene glucose into human erythrocytes in the presence of chlorpromazine relative to the rate in its absence. The points are the mean of between 2–5 observations. The solid line is drawn according to Eq. (2) using the parameters from Fig. 4. The broken line (asymptote) represents the theoretical maximum ratio.

on the diffusion rate constant, k, of ethylidene glucose measured from F(C.V.) plots as described by Widdas.[10] The results have been normalized since there is some variation between individuals.

The rate constant for diffusion, k, is in isotonic units min^{-1} and can be converted to, P, the permeability constant per second using the equation

$$P = \frac{k}{60} \times \frac{\text{cell vol.}}{\text{cell area}} = k \times 0{\cdot}83 \times 10^{-6}.$$

If it is assumed that the cell membrane is made up of a large number of discrete regions with a normal diffusion coefficient D and that there are [M] sites per cm^2

capable of adsorbing CPZ which then alters the diffusion coefficient in the vicinity to D′, it may be assumed that the permeability in the presence of the drug will be given by

$$P = \mathrm{D}([\mathrm{M}] - [\mathrm{MA}]) + \mathrm{D}'[\mathrm{MA}] \tag{1}$$

where [MA] is the concentration of altered sites.

Assuming the reaction of CPZ with the membrane sites follows normal kinetics the affinity constant, K_a, will be given by

$$K_a = \frac{[\mathrm{A}]\,([\mathrm{M}] - [\mathrm{MA}])}{[\mathrm{MA}]},$$

where [A] is the CPZ concentration, whence

$$[\mathrm{MA}] = \frac{[\mathrm{M}]\,[\mathrm{A}]}{[\mathrm{A}] + K_a},$$

Substituting in (1) for [MA] gives

$$P = \mathrm{D}[\mathrm{M}] - \frac{\mathrm{D}[\mathrm{M}]\,[\mathrm{A}]}{[\mathrm{A}] + K_a} + \frac{\mathrm{D}'[\mathrm{M}]\,[\mathrm{A}]}{[\mathrm{A}] + K_a}.$$

Rearranging

$$P = \mathrm{D}[\mathrm{M}]\left(1 - \frac{[\mathrm{A}]}{[\mathrm{A}] + K_a} + \frac{\mathrm{D}'[\mathrm{A}]}{\mathrm{D}[\mathrm{A}] + K_a}\right).$$

Now D[M] is the unaffected permeability which is proportional to the control rate constant, so that;

$$\frac{k}{k_0} = 1 + \left(\frac{\mathrm{D}'}{\mathrm{D}} - 1\right)\frac{[\mathrm{A}]}{[\mathrm{A}] + K_a}. \tag{2}$$

and

$$[\mathrm{A}] = \left(\frac{\mathrm{D}'}{\mathrm{D}} - 1\right)\frac{[\mathrm{A}]}{\frac{k}{k_0} - 1} - K_a. \tag{3}$$

Thus plotting

$$\frac{[\mathrm{A}]}{\frac{k}{k_0} - 1}$$

against [A] should generate a straight line of slope

$$\frac{1}{\frac{\mathrm{D}'}{\mathrm{D}} - 1}$$

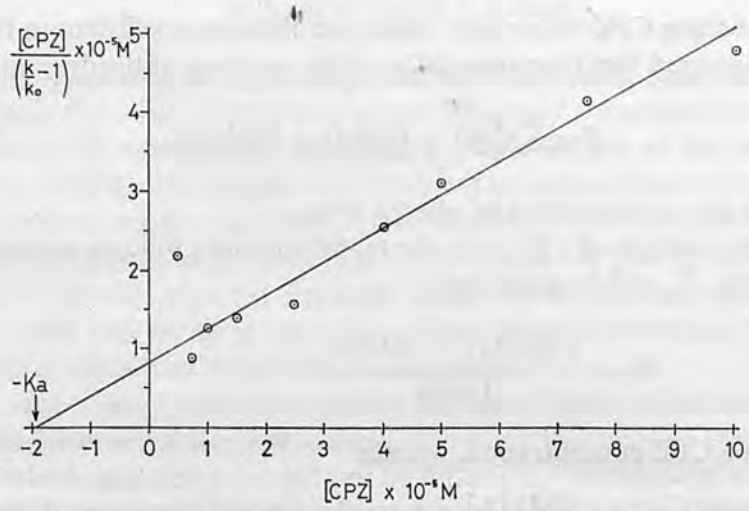

FIG. 4. Data from ethylidene glucose experiments plotted according to Eq. (3). $K_a = 1{\cdot}95 \times 10^{-5}$ M; $D'/D = 3{\cdot}4$. Line fitted by least squares method.

and intercept $-K_a$. This is Fig. 4 and from it K_a is found to be approximately $1{\cdot}95 \times 10^{-5}$ M at 36°.

DISCUSSION

It would seem from these results that CPZ facilitates the passage of molecules such as ethylidene glucose across the membrane and the uptake of drug by the membrane follows, at least as far as this effect is concerned, classic kinetic theory.

It has been shown that CPZ is adsorbed by sites in erythrocyte ghost membranes and that this process is associated with expansion of the membrane and stabilization against hypotonic haemolysis.[17] Adsorption at these stabilizing sites is complete at around 2×10^{-5} and above this the membrane concentration rises steeply. In our experiments there was no evidence of an abrupt change of ethylidene glucose permeability even approaching concentrations that caused spontaneous haemolysis. The affinity constant found by Kwant and Seeman[12] was 6×10^{-6} M at 22°. This may be compared with the present value of $1{\cdot}95 \times 10^{-5}$ M obtained at 36° with intact red cells.

The inhibitory effect on glucose transfer seen at higher concentrations was exhibited by all the phenothiazine derivatives examined. Trifluoperazine was more effective than CPZ, prochlorperazine and promazine which were more effective than promethazine. Owing to the complex nature of the inhibition it is difficult to assign an exact order of potency to these compounds. The related antidepressant compound imipramine also had inhibitory effects intermediate between those of CPZ and trifluoperazine. Nealbarbitone, thiopentone sodium and haloperidol showed no inhibitory effects in the concentration range studied (10^{-7} to 10^{-3} M for barbiturates, 10^{-8} to 10^{-5} M for haloperidol).

The action of CPZ on the transport of glucose across the erythrocyte membrane shows a complex behaviour which must be due to the simultaneous effect of several different actions, the actual degree of any effect being dependent on the concentration. An attempt to explain the changes observed must, therefore, be based on actions which are known to occur at corresponding concentrations.

The initial increase and decrease in rate occur in a concentration range found by Kwant and Seeman[18] to cause the displacement of membrane bound calcium from erythrocyte ghosts. Certainly in our experiments low CPZ concentrations were found to cause clumping of the cells which would be an indication of change in surface charge. One of the functions of calcium in the membrane may be the maintenance of membrane structure, thus loss of calcium would result in a rearrangement of the membrane components and, possibly an alteration of surface charge with a resultant change in transfer rate.

The sharp reduction in exit time seen above 1×10^{-5} M is difficult to account for fully. If, as the results for ethylidene glucose suggest, CPZ alters the membrane structure so that the diffusion of molecules occurs more rapidly the glucose carrier may also be speeded up but this effect is unlikely to be sufficient to explain the three-fold increase in rate that is observed.

From this very high rate of transfer the system is progressively inhibited as the CPZ concentration rises further. As was seen from Fig. 2 this inhibition and the preceeding effects do not involve the glucose-carrier binding site but probably involve a change in the tertiary structure of the carrier or perhaps the surface protein of the membrane.

At still higher concentrations the change progresses to include the binding site, resulting in a decrease in affinity of the carrier for glucose accounting for the increased value of the half-saturation constant.

The acceleration of ethylidene glucose penetration is probably related to the expansion of the cell membrane by CPZ. This may be either a direct effect on membrane lipids[19] or some change resulting from the interaction of CPZ with the surface protein which causes the lipids to be exposed at the membrane surface. Such changes would continue as the concentration increased until finally drug interaction with the membrane protein and lipid produced disruption of the membrane and lysis of the cell.

Many actions of CPZ demonstrate a biphasic effect, low concentrations producing the opposite effect to high ones.[20] CPZ has been shown to inhibit glucose entry into isolated rat spinal cord and muscle[21] whilst experiments in mice have shown an increase in brain glucose which may be explicable in terms of an increased glucose transfer rate across neuronal membranes.[8] These conflicting observations may perhaps be reconciled by our observations. An inhibitory affect on glucose penetration through membranes has been demonstrated in the case of some other C.N.S. depressants, butanol has been shown to inhibit glucose transfer in red cell by optical[22] and isotope[23] techniques although haematocrit methods have failed to confirm this.[24] The inhalation anaesthetics ether, halothane, and methoxyflurane also inhibit glucose penetration into erythrocytes in therapeutic concentrations.[24]

The drugs studied here and exemplified by CPZ have a complex effect on red cell glucose permeability being acceleratory at low concentrations and inhibitory at higher concentrations. If the membranes of brain cell have similar properties the predominant effect must depend on local concentrations but in addition interpretations should bear in mind that brain cells will have a much greater range of surface to volume ratios than red cells and so a concentration of CPZ which was inhibitory would affect first those cells whose glucose utilization was at such a level as to be membrane limited. In this range such an inhibitory action could be contributory to the reduction in the general level of cerebral metabolism which occurs during sedation.

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