THE KINETICS OF SELECTIVE BIOLOGICAL TRANSPORT

III. ERYTHROCYTE-MONOSACCHARIDE TRANSPORT DATA

D. M. MILLER

From the Research Institute, Canada Department of Agriculture, University Sub Post Office, London, Ontario, Canada

ABSTRACT The simplest biological transport system so far extensively investigated is that of monosaccharides in human erythrocytes. Despite its simplicity there is still considerable doubt and divergence of opinion concerning its mechanism. Some confusion may arise as a result of the comparison of diverse data obtained by different workers using a variety of experimental techniques. To minimize this problem, an attempt is made here to repeat, under standard conditions and with as much care as possible, five of the more definitive types of experiments previously performed on this system. It is hoped that the result of this effort is an internally consistent set of data with which the quantitative predictions of various proposed mechanisms may be compared as a primary criterion for their acceptability.

INTRODUCTION

In the search for a mechanistic explanation of selective transport there are four main experimental approaches presently being followed: (1) Attempts are being made to ascertain the molecular architecture of the membrane by such methods as electron microscopy and X-ray diffraction. (2) Model systems approximating the structure and activity of natural membranes are being sought and studied. (3) Attempts to isolate various membrane components so as to determine their chemical nature and biochemical function are now under way. (4) Kinetic studies of intact systems actively engaged in the transport process have been undertaken.

Each of these methods is capable of supplying information which is unique in itself. Consequently none, by itself, is able to provide a total picture of the transport mechanism. Thus, even though we were to succeed in determining the structure of the membrane by methods 1 or 2, we would then have only a static picture of the process and would still have to look to the other methods to provide a final dynamic biochemical mechanism.

The technology of the kinetic method, unlike that of the other three, has been well worked out. In spite of this there does not appear to be a single system for

which a complete set of reliable data is available. The present work was undertaken, therefore, as a modest attempt to provide such data through the study of one system. Many more systems will have to be studied before a comprehensive kinetic description of the transport process is available, but meanwhile, the following data should be useful as a quantitative criteria against which to test theoretical proposals of transport mechanisms.

Probably the main reason extensive studies have not been undertaken in the past is that the systems by which most data were collected are so complex that interpretation is difficult, or so variable that reproducible results are impossible to obtain. In selecting a system for the present work then, the following features were sought: (*a*) *Simplicity*. The greatest simplicity is possessed by a system consisting of two compartments separated by a single membrane. This rules out virtually all tissues and reduces the choice to free-living cells possessing no internal structures (such as vacuoles, mitochondria, nuclei, etc.) or more specifically, to simple bacteria and nonnucleated erythrocytes. (*b*) *Physiological reproducibility*. This is possessed to a high degree by human erythrocytes since they do not grow, sporulate, or divide. Bacteria, on the other hand, must be grown and harvested under exacting conditions in order for them to exhibit a constant response to a given experiment. (*c*) *Simple, stable substrates*. Initially at least, ionized substrates should be avoided since their movements can be complicated by the presence of fixed charges, counterions, and electrical gradients in the membranes. It would also be a distinct advantage if in addition to the substrate a number of competing analogues were available, all in a radioactive form and all resistant to significant chemical breakdown or metabolism within the cell.

Since all these features are possessed by the human erythrocyte-monosaccharide transport system, this system was chosen to obtain the data reported below. Results from five different types of experiments are presented. Most of these experiments have already been performed by other workers and, although there are differences between the absolute values of the measurements reported here and those reported elsewhere, the important feature of this work is that all measurements have been made in one laboratory, by the same workers using the same techniques, so that they should form an internally consistent set of data.

EXPERIMENTAL

Materials

The radioactive sugars were universally labelled ^{14}C compounds obtained from the New England Nuclear Corp., Boston, Mass. All other compounds were reagent grades, and all reducing monosaccharides were found by a gas chromatographic method (1) to contain less than 1% of other sugars as impurities. The erythrocytes were obtained mainly from an outdated blood bank supply. On a number of occasions however, fresh blood was employed with no significant difference observed in the behaviour of cells derived from the two sources.

Isotonic Buffer (IB). IB, used throughout the following experiments, was prepared

by dissolving 7 g NaCl and 3 g Na_2HPO_4 in water, titrating to pH 7.35 with HCl, and diluting to 1 liter. This medium was considered to be approximately isotonic since a given quantity of cells was found to have the same packed volume when centrifuged either from it, or from whole blood.

Preparation of the Cells. Whole blood was centrifuged, the top layer of cells was removed, and the remainder washed once with isotonic NaCl and twice with IB. This same procedure was used to prepare cells for all the experiments described below.

Scintillation Counting Mixture. The mixture consisted of ten parts by volume of a toluene solution of 0.4%, 2,5-diphenyloxazole (PPO), and 0.005% *p-bis*-(0-methylstyryl) benzene, plus eight parts methyl cellosolve. This counting mixture will hold up to 5% water (and an even larger proportion of ethanol) without separation of the components at 0°C.

Procedure

The object of the following experiments was to determine the amount of a particular sugar present in a fixed number of erythrocytes as a function of time, under a variety of conditions. Such measurements were made either during sugar uptake by cells suspended in a solution of the sugar, or during sugar loss by cells previously loaded with the sugar and subsequently suspended in a solution of lower or zero sugar concentration.

Two methods of determining sugar content were employed as follows:

Light Scattering Method. If a penetrating sugar is added to a suspension of red cells, the osmotic pressure of this added compound will produce a rapid shrinking of the cells followed by a slower return to their original volume as the penetrating sugar enters the cell and equalizes the internal and external osmotic pressures. If during this process the intensity of a light beam which has passed through the suspension is measured, it will be found to have varied in direct proportion to the volume (and hence sugar content) of the cells. This phenomenon has been widely used to follow sugar movements in red cells. However, it is restricted to the measurement of the relatively slow movements of a single sugar (thus excluding those in which one sugar is exchanged for another) and is furthermore restricted to experiments with relatively high concentrations of sugars (usualy 100 mM or greater) since volume changes at lower concentrations are usually too small to allow accurate measurements to be made. The light scattering apparatus (LSA) used in this work consisted of a jacketed plexiglass container in which the erythrocyte suspension was held. Light from a light bulb operated by a regulated voltage supply was shone upward through the water jacket and the suspension onto a photovoltaic cell, the output from which was fed directly to a strip-chart recorder. The erythrocytes were prevented from settling by a stirrer placed to one side of the light path. Measurements were made on red cells having a packed volume of 0.1 ml suspended in 60 ml of solution. The apparatus was calibrated, and shown to have a linear response, by measuring the deflection of the recorder pen upon the addition of known amounts of sodium chloride to a cell suspension.

Radioactive Tracer Method. This method involved two main procedures. Firstly, a number of samples were taken (each containing a known quantity of cells) during the course of the sugar movement, and secondly, the cells in each sample were assayed for their sugar content. In detail these procedures were carried out as follows:

Cell sampling. The rates of movement of sugars in some of the experiments to be described below were so rapid that samples had to be taken at intervals of as little as two seconds. This required firstly that the cells be instantaneously dispersed in the test medium to give an accurately known starting time and secondly, that sugar movement in cells contained in subsequent samples be instantaneously terminated after an accurately measured

interval of time. To accomplish this each sample was treated separately, i.e., the cells were dispersed and the sugar movement terminated for one sample before the next was treated. In this way the results from a number of samples, for which different time intervals between dispersal and termination were allowed, could be combined to produce a single record of sugar content vs. time. Each sample was treated in three steps as follows:

Preparation of the cells. A known volume of cells, previously washed and loaded with sugar as required by the experiment, was pipetted as a suspension into each of a number of Hopkins vaccine tubes (sold by most suppliers of laboratory glassware) and centrifuged. The supernatant was then removed as completely as possible by suction through a fine tube, leaving only the packed cells in the bottom few millimeters of the capillary portion of the vaccine tube. A small amount of the medium in which the cells were to be suspended was then added to the top part of the vaccine tube and removed by lowering the suction tip to within a millimeter or two of the cells. In this way the vaccine tube was rinsed by the medium passing down the capillary and out the suction tube without disturbing the cells. Once more the medium was placed in the top part of the vaccine tube which was then brought to 20°C in a water bath.

Dispersion of the cells. In this step an automatic syringe is required, i.e., a syringe whose depressed plunger when released is forced up by a spring until arrested at a predetermined volume by an adjustable stop. The syringe was fitted with a 3 inch No. 17 stainless steel needle (the tip of which had been ground to a broad "V" when viewed from the side), set to a volume of 1 ml, and brought to 20°C by placing it in a tube immersed in the bath. The experiment was started by depressing the plunger of the syringe, then pushing the tip of the needle to the bottom of the vaccine tube, while simultaneously releasing the plunger and starting a stopwatch. With the first movement of the plunger, the cells, together with some of the medium, were swept up the needle and into the syringe with such speed and turbulence that mixing was virtually instantaneous. The remaining movement of the plunger brought the rest of the medium into the syringe mixing it thoroughly as it entered.

Termination of the sugar movement and assay of the sugar content. After the required length of time, during which the filled syringe was maintained at 20°C, the sample was delivered by forcefully depressing the plunger. The sugar movement was terminated either by filtration or by quenching the sugar movement with an inhibitor as follows:

(*a*) *Filtration.* The sample was ejected into 5 ml cold saline which was being sucked through a one inch glass fiber filter disc (Gelman Instrument Co., Ann Arbor, Mich.). After all the liquid had been sucked through, the wet disc was placed directly into a scintillation vial, dried under a stream of warm air, 5 ml counting fluid added, and assayed by a scintillation counter. Red cells tend to hemolyze during filtration but this could be reduced by using a small volume (1 μl) of cells in each sample and by making the saline four times isotonic.

(*b*) *Quenching.* The procedure for the use of mercuric chloride to inhibit sugar movements in erythrocytes as outlined by Lefevre and McGinniss (2) has been widely used and appears to be rapid and complete at high concentrations. Levine and Stein (3) have reported that the addition of iodide to the mercury quenching solution improves inhibition, particularly at low concentrations. Therefore, in the present work the original solution was used for experiments involving sugar concentrations greater than 100 mM and Levine and Stein's solution was used at lower concentrations.

The contents of the syringe which contained 10 μl cell volume were squirted directly into 10 ml ice cold quenching solution held in a conical centrifuge tube. This was centrifuged at 4000 *g* for 5 min, the supernatant poured off, centrifuged again briefly to drain the sides of the tube, and the residual supernatant removed by suction. During this entire procedure the tube and contents were kept at 0–4°C. The tube was then warmed to room temperature,

50 μl isotonic NaCl added, and the cells suspended with the aid of a stirring rod and vortex mixer. Next, 1.20 ml 95% ethanol was added, mixed, the stirring rod removed, and the tube centrifuged to settle the residual material. Exactly one ml supernatant was withdrawn, placed in a scintillation vial together with 10 ml counting mixture, and counted. Counting efficiency was found (through the use of internal standards) to be 70% under these conditions.

Both methods were employed on certain experiments and found to agree within the limits of error. The quenching method however provided superior reproducibility and consequently all the radioactive results given below were obtained by this method.

For some of the experiments to be described, the time interval between samples was 15 sec or longer so that a slightly different procedure could be employed as follows: A vaccine tube containing 0.1 ml prepared cells packed in its capillary portion and 10 ml suspending medium present in its upper portion was brought to 20°C in a water bath. At zero time the cells were taken up by the automatic syringe and ejected back into the medium in the tube. With the end of the needle still in the medium, the plunger was then worked up and down to provide thorough mixing. Next a series of 1 ml samples were withdrawn at regular intervals, again using the syringe, and injected into the quenching solution after which they were treated as previously described.

Many of the experiments were performed to obtain the initial rate of movement of sugar, i.e., the rate with which the sugar was either taken up, or lost by the cells at zero time. In this case, the sugar content was plotted as a function of time and if, as was frequently the case, the early portion of the curve was straight, then those points on the straight portion were subjected to a least squares analysis to give the slope at this point. Otherwise a smooth curve was drawn through the points and a tangent to this curve drawn by eye at the time = 0 intersection of the curve. For loss experiments this tangent (or straight line portion) had a negative slope which, when extended, cut the time axis at a point which represented the amount of time required for all the sugar present in the cells at zero time to escape at the initial rate. For uptake experiments the tangent had a positive slope so it was necessary to follow the course of the sugar uptake until it reached a constant value. This value was then projected backward, parallel to the time axis, until cut by the tangent and the time at which this occurred represented that required for the cells to become equilibrated with the suspending medium at the initial rate. Thus, dividing the concentration of the medium by the time interval gave the initial rate of uptake.

Red cells are known to have a constant surface area for all volumes except those near the hemolytic volume. This area then, is constant for any given number of cells, so that we may choose any quantity as a standard. For convenience, the quantity chosen in this work is that number of cells whose isotonic cell water volume is 1 liter. This quantity (referred to as a cell unit) need not actually be determined however, since when loading the cells it is assumed that the concentration of the sugar in the cell water at equilibrium reaches that of the external solution. Provided the buffer is isotonic, this means that if the concentration of sugar in the loading solution is x moles/liter, then a cell unit will contain x moles of sugar irrespective of any subsequent change in its volume due to osmotic water movements. Now if Δt is the time required for the internal sugar concentration to reach that outside the cell at the initial rate, then that rate must be $x/\Delta t$ mmoles/sec/cell unit (or mmoles/sec/liter isotonic cell water).

RESULTS

The amount of supernatant occluded by the packed cells during centrifugation in the radioactive method was found by the use of ^{14}C inulin to be about 3% of the cell volume. The amount of radioactivity from the suspending medium which was

retained in this way by the cells, however, was only 0.3% since the sample was diluted ten times by the quenching solution before centrifugation. This quantity was constant throughout any given experiment and therefore had no influence on the *slope* of the rate curves.

TABLE I

GLUCOSE LOSS VS. EXTERNAL GLUCOSE CONCENTRATIONS

Concentration	0	2.5	5.0	7.5	10	20	30	40
Rate	100 ±9 (5)	45 ±3 (6)	26 ±0.6 (4)	20 ±0.6 (6)	16 ±0.2 (3)	7.3 ±0.4 (5)	5.3 ±0.3 (4)	3.5 ±0.5 (4)

The initial rate of loss of radioactive glucose from human red cells previously equilibrated with a 130 mM glucose* solution, suspended in a solution of the same sugar at the concentrations indicated. Concentrations are in mM and rates in mmoles/min/liter isotonic cell water. Temp. 20°C, pH = 7.35.

TABLE II

SORBOSE TRANSPORT INHIBITION

Inhibitor—glucose					
Concentration	0	12.5	25.0	37.5	50.0
Rate of loss	4.7 ± 0.7 (6)	2.4 ± 0.2 (3)	1.7 ± 0.2 (4)	1.3 ± 0.1 (5)	1.10 ± 0.05 (4)
Rate of uptake	4.8 ± 0.8 (11)	2.6 ± 0.6 (4)	2.1 ± 0.4 (4)	1.7 ± 0.4 (4)	—
Inhibitor—mannose					
Concentration	0	20	40	60	
Rate of uptake	4.8 ± 0.8 (11)	3.3 ± 0.1 (4)	2.34 ± 0.04 (4)	1.7 ± 0.1 (4)	
Inhibitor—galactase					
Concentration	0	25	50	75	
Rate of uptake	4.8 ± 0.8 (11)	3.3 ± 0.2 (4)	2.1 ± 0.1 (4)	1.6 ± 0.1 (4)	

The initial rate of transport of sorbose as a function of an inhibiting sugar concentration. *Uptake experiments:* The cells were equilibrated with the inhibitor at the concentration indicated and a solution of sorbose added to a concentration of 230 mM. The rate of sorbose uptake was then measured by the light-scattering method. *Exit experiments:* The cells were first equilibrated with sorbose at 230 mM, then glucose was added to the required concentration, and the cells again equilibrated with this solution. The cells were centrifuged and at zero time resuspended in a solution containing the inhibitor at the required concentration plus 230 mM sucrose to balance the osmotic pressure. The loss of sorbose was measured by light scattering. Concentrations in mM; rates in mmoles/min/liter isotonic cell water. Temp. 20°C, pH = 7.35.

Considerable care has been exercised in the performance of these experiments. A temperature of 20°C, rather than the normal temperature of 37°C, was used throughout so that the more rapid movements were slowed to allow more accurate timing. At least three measurements (and usually more) were made on each value reported. This made it possible to discard spurious results and to provide a reasonably reliable standard deviation in each case. The ratio of cell volume to that of the suspending medium was 1 % or less in all measurements. This allows us to consider the external concentration as constant.

TABLE III

RATES OF EXCHANGE BETWEEN DIFFERENT SUGAR PAIRS

Inner sugar	Outer sugar	Rate
Glucose*	None	100 ± 9 (5)
Glucose*	Glucose	190 ± 20 (7)
Glucose	Glucose*	190 ± 20 (4)
Glucose*	Mannose	260 ± 2 (4)
Glucose*	Galactose	250 ± 20 (4)
Mannose*	None	95 ± 5 (8)
Mannose*	Mannose	170 ± 10 (10)
Galactose*	None	80 ± 7 (10)
Galactose*	Galactose	125 ± 10 (9)

The initial rate of exchange of radioactive with nonradioactive sugars. Cells were loaded with the sugar listed as the inner sugar at a concentration of 130 mM and transferred to a solution either free of sugar or containing a second sugar (outer sugar) also at 130 mM. One of the sugars in each case was radioactive and its initial rate of transport (in mmoles/min/liter isotonic cell water) is reported. Temp. 20°C, pH = 7.35.

A description of the types of experiments and the results obtained from them now follows. Note that the tables in this section contain in order, the average values measured, their standard deviations, and in brackets, the number of observations made.

Type I Rate of Loss of Glucose into Solutions of Low Glucose Concentration

This type of experiment was first performed by Sen and Widdas (4) and used by them to determine the constants in the simple carrier theory. In the present work, cells were equilibrated with 130 mM glucose solution, centrifuged, resuspended in a solution of the same sugar at lower concentration, and the initial rate of loss of the sugar determined by the radiotracer method. Table I lists the results from these experiments.

Type II Glucose Inhibition of Sorbose Transport

Inhibition of sorbose transport has also been used as a method of determining the affinity constant of glucose for the transport sites (3, 5). This is based on the idea

that when half the sites are occupied by glucose, the rate of transport of sorbose should be half of what it is in the absence of glucose. Thus by plotting the rate of movement of sorbose either into or out of red cells against glucose concentration, we can determine the concentration of glucose which gives half maximal sorbose transport, and this should be its half saturation or affinity constant. The results of these types of experiments are given in Table II.

TABLE IV

RATES OF EXCHANGE OF GLUCOSE VS. CONCENTRATION

Concentration	19.0	29.6	38.0	66.5	130
Rate	85 ± 5 (4)	120 ± 15 (5)	130 ± 13 (5)	180 ± 7 (6)	190 ± 20 (7)

The initial rate of loss of glucose* from loaded cells into nonradioactive glucose solutions as a function of sugar concentration. The indicated glucose concentrations are the same in the cells and in the suspending medium and are expressed as mM. The rates are given in units of mmoles/min/liter isotonic cell water. Temp. 20°C, pH = 7.35.

TABLE V

COUNTER TRANSPORT MEASUREMENTS

Time	0.5	1.0	1.5	2.0	3.0	4.0	∞
Uptake	8.9 ± 0.8 (3)	10.8 ± 0.8 (4)	9.6 ± 0.5 (4)	7.8 ± 0.5 (4)	4.1 ± 0.4 (4)	2.1 ± 0.3 (4)	1.0

The amount of glucose* taken up by cells previously equilibrated with 130 mM nonradioactive glucose as a function of time (in minutes) from a solution of 4.3 mM glucose*. The uptake is expressed as a ratio of the amount of glucose* present in the cells at the indicated time relative to that at equilibrium (infinite time). Temp. 20°C, pH = 7.35.

Type III Initial Rate of Exchange Between Various Sugar Pairs

Recently a number of workers (3, 6, 7) have shown that the rate of loss of sugars from red cells is more rapid when a sugar is present outside the cells than when it is absent. This type of experiment is usually performed by loading the cells with a radioactive sugar and then transferring them to a solution of nonradioactive sugar at the same concentration after which the rate of loss of the radioactive sugar is measured. In Table III a number of such experiments using different sugar pairs is listed. Also in Table III is an experiment in which uptake is measured, i.e., the rate of entry of radioactive glucose (glucose*) into cells loaded with nonradioactive glucose is reported. Uptake measurements into sugar-free cells can not be made using the present procedures since the concentration of radioactive sugar rises so rapidly that the rate of outward transport approaches that of uptake, drastically reducing the apparent rate of entry. This was not a problem with sugar-loaded cells since competition by the nonradioactive sugar prevented outflow of the radioactive sugar

during a considerable period at the beginning of the experiment during which measurements could be made.

Type IV Initial Rate of Exchange of Glucose as a Function of Concentration

This is similar to Type III experiments. The cells were loaded with glucose*, transferred to a nonradioactive glucose solution of the same concentration, and the initial rate of loss of glucose* measured. Similar measurements were made over a range of glucose concentrations.

Type V Counter Transport Experiments

Experiments of this type had been performed previously by the author on mannose and galactose (8) and were repeated here using glucose. The procedure was to load cells with a high concentration of nonradioactive sugar, transfer them to a solution having a low concentration of glucose*, and then to follow the increase of radioactivity within the cells. In Table V, where these results are reported, it can be seen that the cells temporarily attained a concentration of glucose much higher than that at equilibrium, caused by the presence of the nonradioactive sugar within. The second sugar was, of course, flowing out as glucose* entered (hence the name "counter transport"), and when the latter had completely left, the glucose* reached its final equilibrium value. A quantitative description of this complex process should provide a considerable challenge to any proposed mechanism.

DISCUSSION

It might be argued that in selecting the erythrocyte-monosaccharide transport system we have chosen one of such profound simplicity that any conclusions reached from this study will bear slight relationship to any other system. This might be especially true since this system does not exhibit active transport, that is, the substrate does not move in a direction opposite to its concentration gradient through the agency of energy supplied by the cell. For sugars in general, however, active transport appears to be the exception (9) and even when it does occur it can be eliminated, in one case at least (10), by metabolic inhibitors, leaving a residual system having kinetically the same characteristics as the erythrocyte-monosaccharide system. This suggests the possibility that there are present in all cells basic and probably primitive, nonactive, but selective transport systems allowing the entry of required substances while unwanted compounds are excluded. To some of these systems there appears to have been added, under competitive pressure, an active component aiding the cell to retain those metabolites in short supply which happen to have entered it via the nonactive route. It is because this possibility exists, therefore, that a study of the erythrocyte system is important.

In the next publication of this series (11) a number of proposed mechanisms will be subjected to kinetic scrutiny. However, before using the above data in this way

it should be understood that a kinetic analysis simply provides a mathematical description of the process under investigation, and while we may discard a mechanism which fails to correspond to this description, it does not follow that one which does is necessarily correct since others might also be found to provide an adequate mathematical correspondence. Nevertheless, the more varied the kinetic evidence, the more stringent the mathematical requirements, and the fewer the mechanistic possibilities. Hence the variety of types of experiments presented here.

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