Evidence for Carrier-Mediated Transport of Monosaccharides in the Ehrlich Ascites Tumor Cell

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ABSTRACT Evidence for carrier-mediated transport of monosaccharides in the Ehrlich ascites tumor cells was provided through kinetic analysis of data obtained by: (a) studying sugar uptake by dilute cell suspensions with an optical densimetric apparatus, (b) studying sugar uptake by thicker cell suspensions by means of direct chemical analytical methods using packed cell plugs, (c) observing the effects of a competitive inhibitor upon sugar uptake with the chemical analytical method, and (d) measurement of tracer uptake of a high affinity sugar in thick cell suspensions in the absence of net movement. Quantitative application of the data obtained with the above experimental procedures to theoretical model systems derived for both carrier-mediated transport and simple passive diffusion indicated that the results were consonant with predictions for the carrier-mediated transport model, but could not be explained on the basis of uncomplicated diffusion.

Several lines of evidence have led recurrently to the proposal that cell membranes include components that mediate the transfer of certain classes of substances through the lipid protein membrane which, by virtue of its physicochemical characteristics, would not otherwise be expected to permit the passage of many of these substances.

The transport model adopted here to interpret the movement of sugars through the ascites tumor cell membrane involves a "carrier" mediation, and was first suggested by Widdas (1) for the transport of sugars across the placental membrane, and has been applied by Park et al. (2) and by LeFevre and his associates (3–6) to the transport of sugars through the human erythrocyte membrane. This and similar "equilibrating" transport systems differ from uncomplicated passive diffusion in that they exhibit "saturation" kinetics, high substrate stereospecificity, competition for transport between structurally similar molecules, and special kinetic phenomena discussed.
below, but do not qualify (by definition) as active transport systems, in that there is no accumulation of substrate against an electrochemical gradient.

Widdas's model (1) emphasized the mobility of the carrier, the rate-limiting step in the transport being the diffusion of the carrier-sugar complex through the membrane, in which the carrier itself is assumed to be confined. The uphill transport of a competing sugar (a phenomenon which requires that the reacting site be mobile) has been observed in the human erythrocyte (7). LeFevre and McGinniss (5) found that the speed of equilibration of tracer glucose (added after completion of chemical equilibration with a high concentration of nontracer glucose), relative to the speed of the prior chemical equilibration, was on the order of 50–100 times greater than would be predicted for an uncomplicated diffusion process. This finding is in accordance with the kinetic behavior predicted for a mobile carrier system with a high affinity sugar.

Dissociation constants for various sugars estimated in the human red cell system by kinetic analysis of competitive inhibition by β-(p-hydroxyphenyl)-phlorpropiophenone (phloretin) (8) were consistent with the sequence of affinities of the same sugars as determined by mutual competition for transport among pairs of monosaccharides (9). Later, it was suggested that this sugar affinity sequence reflects an explicitly conformational stereospecificity with respect to the transported sugar (4).

Countertransport of sugars has been observed for cardiac muscle (2), certain bacteria (10), blood-CSF barrier (11), and skeletal muscle (12). The countertransport phenomenon for monosaccharides has been demonstrated in ascites tumor cells (13); this argues for the involvement of a mobile carrier entity. Also, some experimental evidence has appeared to exclude simple passive diffusion as the transport mechanism (14, 15), but no satisfactory kinetic analysis suggesting any particular transport model has been carried out. It will be shown in this study that the data derived from the several experimental approaches not only are in good accord with the carrier hypothesis, and specifically with the Widdas model system, but also imply a close identification of this system with that described for the mammalian erythrocyte.

TECHNICAL

The Ehrlich ascites tumor used was obtained from Dr. T. Hauschka (Roswell Park Memorial Institute, Buffalo, N.Y., Lettre D strain). The cells were prepared in essentially the same manner for each of the three phases of study. The tumor was transferred every 10 days by intraperitoneal inoculation of 0.2 ml of ascitic fluid into white female Swiss mice, and harvested by aspiration with a syringe by intraperitoneal puncture. The cells were collected into isotonic, buffered medium (16), and washed once or twice in about 50 ml of isotonic medium per ml of ascitic fluid. Preparations containing large numbers of red blood cells were discarded. The cells were resus-
pended in isotonic medium before use (0.5% cells, by volume, for densimetric experiments, and about 20% cells for chemical uptake experiments). Anticoagulant substances were not added to the cell suspension, but calcium salts were omitted from the bathing solutions.

1. Densimetric Techniques

For experiments involving the use of an optical system for detection of changes in cell volume, the 0.5% cell suspension was kept gently stirred in a plastic vessel maintained at 37°C. The densimetric instrument used was a modification of the system of Ørskov (17). The uptake process was recorded by placing a volume of cell suspension in an optical cuvette, stirring rapidly, and adding penetrant sugar. The continuous deflection due to the penetration of sugar and accompanying water was recorded. The relation between osmotic activity and galvanometer deflection was studied by recording the deflection resulting from the addition of varying concentrations of NaCl (nonpenetrant) to aliquots of cell suspension.

2. Chemical Uptake Techniques

A more direct method of determining sugar uptake, by measuring the penetrant sugar content of packed cells at various times after the introduction of the sugar, was used in conjunction with the optical method. The sugars were the same as those used in the experiments described above, and the inhibitors used were diethylstilbestrol and phloretin, both potent inhibitors of sugar transport in the human erythrocyte (3).

Sugar uptake was followed by adding a small volume of sugar solution to an equal volume of thick cell suspension (generally about 20% cells by volume). At the desired times, the transport was stopped by rapid transfer of part of the suspension (exactly the same volume for each sample) to a tenfold larger volume of ice-cold (4°C), twice isotonic medium containing mercuric chloride at 2 mM, and high speed centrifugation in the cold for 3 min. The supernatant liquid was immediately decanted for later analysis, and the inside of the centrifuge tubes above the cell plugs blotted dry. The 2mM mercury and the low temperature of the "fixer" solution act to stop, or fix, the transport (18) so that the measured incubation time could be taken as the interval between the addition of sugar and contact with the iced solution. A small volume of hemoglobin solution was added to the cell plugs and to volumes of the supernatant in order to adsorb the residual mercury (which otherwise interfered variably with the chemical analysis for sugars (6)). Somogyi filtrates (19) were prepared from the hemoglobin-treated packed cell plugs and volumes of supernatant, and the sugar content of all samples was determined by procedures slightly modified from the methods of Nelson (20) and Roe et al. (21).

A correction for the sugar content of the extracellular fluid trapped in interstices between the packed cells of the plug was made by incubating mannitol-14C (a substance which presumably distributes itself uniformly in the extracellular space, but which does not penetrate into, nor adsorb to the cells) in parallel samples of cell suspension for varying periods of time (equivalent to the incubation times for the sugar-containing samples). The mannitol-containing samples were treated in exactly the same manner as described for the other samples. Radioactivity was determined
using a toluene solution of scintillator, ethanol, and a 1 ml aqueous sample. Counting was performed with a Tracerlab LSC-10B liquid scintillation counter (Tracerlab Inc., Lyndhurst, N.Y.); counts were at least 20X background. After extensive unsuccessful experience with other methods for the determination of extracellular space, the above method of parallel incubation with mannitol-14C was adopted as the only procedure giving acceptably reproducible results in our hands.

Uptake curves were constructed from the measured cell sugar content at various incubation times, and the half-times of entry estimated visually from the graphs.

3. Inhibitor Studies
Experiments involving diethylstilbestrol, phloretin, and phloridzin were conducted in a manner similar to the chemical uptake experiments, except for the addition of the inhibitor. Commercial preparations of these agents were dissolved in ethanol and added to the cell suspension (0.01 ml of ethanolic solution per ml cell suspension) several minutes before adding the penetrant sugars. However, the one inhibitor which produced meaningful results (diethylstilbestrol) did not at effective concentrations actually form a true solution in the aqueous cell suspension, having very low solubility in a 1% ethanolic solution. It is likely that the unusual degree of scatter in the experimental data here is due to the failure to achieve a true solution of the inhibitor. Each experiment included samples incubated with both sugar and inhibitor, and with sugar only (all at the same sugar concentration). The procedure was as described above for chemical uptake studies.

4. Tracer Flux Studies
Cell suspensions for the tracer flux studies were prepared in the same way as for the chemical studies, and sampled similarly. These samples were divided into two groups and to each of one group was added an identical volume of a solution of unlabeled D-xylose. The remaining samples were incubated with the same sugar solution for various periods of time, in order to establish the course of net chemical uptake. After equilibrium between cells and external medium had been attained in the first set of samples, a small volume of a solution of tracer D-xylose-14C was incubated for various periods of time with each of the equilibrated samples. This tracer solution was supplemented with nontracer D-xylose such that the concentration of sugar in the mixture was the same as the final concentration of D-xylose in the previously equilibrated samples to which it was added. Since tracer equilibration was expected to occur within a relatively short period of time, the incubation periods chosen were brief, and sampling was performed quickly. Extracellular volumes were separately determined with mannitol-14C as described above. Radioactivity was in these instances measured with a Packard Tri-Carb liquid scintillation system (Packard Instrument Co., Downers Grove, Ill.), using the same scintillation mixture as mentioned above. After correction for trapped total and tracer xylose, the half-times of net uptake and tracer equilibration were determined from their respective sugar content vs. log time curves.

THEORETICAL
The contrasting kinetics predicted for the mobile carrier facilitated diffusion model (Fig. 1), and for uncomplicated diffusion have been developed elsewhere, notably by
Rosenberg and Wilbrandt (22), LeFevre and McGinniss (5), and Widdas (23). With passive diffusion, involving only passage along an aqueous pore, or through the homogeneous membrane substance, it is assumed that there exists a constant activity gradient (approaching the steady state) and independent movement of solvent and solute. Under these conditions, the flux in either direction is proportional to the respective activities of the solute on either side of the membrane, and the net inward movement is given by:

$$\frac{dS}{dt} = k_d [C_o - C_i],$$

where $S$ is the quantity of intracellular penetrant, $C_o$ is the concentration of penetrant in the outside medium, and $C_i$ is its concentration inside the cells. The constant $k_d$ is determined by the diffusivity of the penetrant through whatever paths it takes through the membrane, and the number and dimensions of such paths. If the concentrations are expressed in osmotic units (1 osmole is taken to be 330 milliosmoles), and $S$ is given in units equivalent to the cell’s natural content of osmotically active material, $k_d$ is expressed as isovolumes/minute.

In contrast to this unencumbered diffusion, the carrier-mediated transport model described by Fig. 1 (identical to the type “E” described by Rosenberg and Wilbrandt...
(22) and adopted by LeFevre and McGinniss (5) from the model originally proposed by Widdas (23)) involves a transient reaction at each membrane interface of the penetrant molecule (S), with some freely diffusible component (C) of the membrane, diffusion of the resulting carrier-penetrant complex (CS) through the membrane, and dissociation at the other interface. It is assumed (a) that the rate-limiting step is the diffusion of the carriers and the complex through the membrane, the reactions at the interfaces being relatively rapid and always nearly at equilibrium, (b) that free penetrant exists only in the two aqueous compartments inside and outside the membrane, the carrier and carrier-penetrant complex being confined to the membrane, (c) that the total amount of carrier present in the membrane is fixed, and (d) that there is within the membrane a linear gradient for CS and for free C (approaching the steady state). Thus the fraction of carrier which is complexed as CS at the aqueous interfaces is $C_o/(C_o + K)$ on the outside, and $C_i/(C_i + K)$ on the inside (since $K = k_1/k_2 = k_3/k_4$), where $K$ is the carrier-penetrant complex dissociation constant.

The flux in a given direction would be presumed to be proportional to the fraction of combined carrier on the input side, and to the fraction of uncombined carrier returning from the output side. The net transport (the difference between the two oppositely directed fluxes) should be described by the following equation:

$$\frac{dS}{dt} = k_e \left[ \left( \frac{CS}{C_{r_o}} \right) \left( \frac{C}{C_{r_i}} \right) - \left( \frac{CS}{C_{r_i}} \right) \left( \frac{C}{C_{r_o}} \right) \right]. \quad (2)$$

where $C_r$, the total amount of carrier at each interface, is equal to the local $C + CS$ and $k_e$ is a constant representing the diffusivity of the carrier-penetrant complex and the fixed concentration of carrier sites present (dimensions are quantity/time). Substitution for $C_i$ and $C_o$ gives:

$$\frac{dS}{dt} = k_e \left[ \frac{CS}{C_{r_o}} - \frac{CS}{C_{r_i}} \right],$$

or

$$\frac{dS}{dt} = k_e \left[ \frac{C_o}{C_o + K} - \frac{C_i}{C_i + K} \right]. \quad (3)$$

In application to the actual experiments, cell water content is a sufficiently small part of the total suspension water that relatively little error is introduced by treating $C_r$, the external penetrant concentration, as a constant. However, the varying $C_i$ must be expressed as a function of $S$ by reference to the varying cell water volume, $V$ (i.e., $C_i = \frac{S}{V}$). If the cell behaves as an osmometer, the volume $V$ is directly proportional to the inverse of the internal osmotic activity; and since water equilibrates across the membrane much more quickly than the transported solutes, the inside and outside osmotic activities may be considered always equivalent. Thus the changing
cell volume is always quantitatively related to the solute transport, in accordance with the relation:

\[ V = \frac{C_m V_i + S}{C_m + C_o}, \]

where \( C_m \) is the concentration of isosmotic solute originally present inside the cell (equal to the isotonic concentration of the nonpenetrant ingredients in the external medium), and \( V_i \) is the cell water volume under isotonic conditions. (Both \( C_m \) and \( V_i \) reduce to unity if the isotonic units of concentration and quantity are used.) After substitution for \( V \) and integration (setting the initial conditions: \( S = 0 \) when \( t = 0 \), to correspond to the experimental arrangement whereby the cells were initially sugar-free), equations 1 and 3 reduce to:

\[ k_{d1/2} = (1 + C_o) \ln \frac{C_o}{C_o - S} - S, \]  

(1a)

and

\[ k_{d1/2} = \frac{(K + C_o)}{K} \left( (C_o + K)(1 + C_o) \ln \frac{C_o}{C_o - S} - S(1 + C_o + K) \right). \]  

(3a)

The half-time \( (t_{1/2}) \) is defined as the time at which \( S = S_o/2 \), where \( S_o \) is the equilibrium value of \( S \) (when \( t = \infty \)). But since \( S_o = V_o(C_m + C_o) - C_m V_i \), and since \( C_m \), \( V_o \), and \( V_i \) are all unity, \( S_o \) is numerically equal to \( C_o \). Consequently, the half-time expressions for uncomplicated diffusion and for the mobile carrier system, respectively, are:

\[ k_{d1/2} = 0.693 + 0.193C_o \]  

(1b)

and

\[ k_{d1/2} = \frac{C_o + K}{K} \left[ 0.693K + 0.193C_o(1 + C_o + K) \right]. \]  

(3b)

Comparison of these two equations clearly marks the vast difference in half-time dependency upon penetrant concentration that is possible with the two systems. Fig. 2a illustrates the relations predicted by simple passive diffusion (heavy line) and the mobile carrier model at high saturation (light line). Fig. 2a may be compared with the observed behavior (Fig. 2b).

For examination of the data obtained by densimetric measurement of the time course of cell volume change during sugar uptake, it is desirable to reexpress equations 1 and 3 so that the variable is \( V \), instead of \( S \).

This is accomplished by substituting

\[ V(C_m + C_o) - C_m V_i \]  

for \( C_i \), and \( (C_m + C_o) dV \) for \( dS \)

in equations 1 and 3, and integrating the initial volume \( V \), being \( \frac{C_m V_i}{C_m + C_o} \) to give
FIGURE 2a. Alternative theoretical predictions between uptake half-times and external penetrant concentration, by diffusion (heavy line), and by carrier-mediated transport under conditions of high saturation (light line). See text for details.

FIGURE 2b. Actual half-times of entry of various sugars into suspensions of ascites tumor cells plotted against the external penetrant sugar concentration.
for simple diffusion, and

\[
k_d = (1 + C_o) \left[ \ln \frac{1 - V_o}{1 - V} - V + V_o \right]
\]

for the carrier model system. These relations, of course, yield the same half-time identities as those given by equations 1 b and 3 b, upon appropriate substitution of the halfway volume:

\[
V_{1/2} = \frac{V_o + V_e}{2} = \frac{2 + C_o}{2 + 2C_o}.
\]

For the interpretation of the behavior in the presence of a competitive inhibitor, \( I \), it has been assumed that \( I \) combines with the carrier, \( C \), only at the outside of the cell membrane and is not transported. Then, \( C_{r_i} = C_{S_o} + CI_o + C_o \); and \( C_{r_i} = C_{S_i} + C_i \). Thus, if \( K_i = \frac{IC}{CI} \) and if the flux in either direction through the membrane is proportional to the product of the fractions of combined carrier on one side and uncombined carrier on the other (as for equation 2), then substitution for \( C_i, CS_i \), and \( C_r \) leads to the following expression for the transfer rate in the presence of the inhibitor:

\[
\frac{dS}{dt} = k_e \frac{KK_i(C_o - C_i)}{(C_oK_i + IK + KK_i)(C_i + K)}.
\]

Substitution for \( C_i \) and integration as before (treating the inhibitor concentration in the external medium, \( I \), as a constant) yield:

\[
k_d = \left( \frac{I}{K_i} + \frac{C_o}{K} + 1 \right) \left[ (C_o + K)(1 + C_o) \ln \frac{C_o}{C_o + S} - S(1 + C_o + K) \right].
\]

This equation leads to the following expression for the half-time of entry of penetrant when an inhibitor acts upon the system:

\[
k_{d/2} = \left( \frac{I}{K_i} + \frac{C_e}{K} + 1 \right) [0.693K + 0.193C_o (1 + C_o + K)].
\]

Equation (4 b) is used to assign a value for \( K_i \) on the basis of observed half-time prolongation by an inhibitor (when \( K \) has been established by other means), and this can then be applied to predict the magnitude of inhibition for sugar of varying affinity constants.

The carrier mediation hypothesis predicts that when cells are incubated with high
concentrations of a high affinity sugar, the ratio of the rate of gross chemical equilibration to subsequent tracer equilibration will be much less than the ratio predicted by uncomplicated diffusion (4). For the experimental situations with tracer xylose in this study, $C_o = C_i$, $S = S_e$, and $V = V_e$ throughout. If:

- $T_i$ is the concentration of tracer inside the cells at any time $t$,
- $T_o$ is the concentration of tracer present in the external medium, and will be treated as essentially a constant for the same reasons that $C_o$ is treated as a constant in previous equations,

then, $T_{ie}$ (concentration of tracer inside the cell at equilibrium) is equal to $T_o$, and $S = S_e$ (since these cells have been equilibrated with penetrant sugar prior to addition of tracer).

The net movement of tracer is simply the difference between tracer influx and efflux, and in the case of carrier-mediated transport, it is given by the following expression:

$$\frac{dT_i}{dt} = k_o \left( \frac{C_o T_o}{C_o^2 + K C_o} - \frac{C_i T_i}{C_i^2 + K C_i} \right),$$

or,

$$\frac{dT_i}{dt} = \frac{k_o}{C_o + K} (T_{ie} - T_i). \quad (5)$$

Integration of equation (5) ($T_i = 0$ when $t = 0$ since no tracer is initially present inside the cells) gives:

$$k_d = (C_o + k) \ln \frac{T_{ie}}{T_{ie} - T_i},$$

or,

$$\frac{T_i}{T_{ie}} = 1 - e^{-k_d t/(C_o + K)}. \quad (5a)$$

Substitution of 0.5 $T_{ie}$ for $T_i$ when $t = t_{1/2}$, gives for the half-time situation:

$$k_d t_{1/2} = 0.693 (C_o + k). \quad (5b)$$

For the case of uncomplicated diffusion, the corresponding relations are simply:

$$\frac{dT_i}{dt} = k_d (T_o - T_i),$$

or,

$$\frac{dT_i}{dt} = k_d (T_{ie} - T_i). \quad (6)$$
whence

\[ k_{\Delta t} = \ln \frac{T_{te}}{T_{te} - T_i}, \quad (6a) \]

and

\[ k_{d_{1/2}} = 0.693 \quad (6b) \]

at any \( C_o \).

Thus the ratio of half-times (net movement/tracer equilibration) would be only

\[ \frac{0.693 + 0.193 C_o}{0.693} = 1 + 0.279 C_o \]

for a diffusion system, whereas for the carrier model, it would be:

\[ \frac{C_o + K}{K} \left[ 0.693 K + 0.193 C_o (1 + C_o + K) \right] \]

\[ \frac{0.693(C_o + K)}{1 + 0.279 C_o \left( 1 + \frac{1 + C_o}{K} \right)} \]

The latter figure can, of course, become very large at a reasonable \( C_o \) with a high affinity sugar (small \( K \)).

**DATA ANALYSIS AND RESULTS**

**Densimetry** The Ørskov optical recording system is useful when galvanometer deflection can be shown to be related to the movement of the penetrant molecule into (or out of) the cells. Water equilibration across the ascites tumor cell membrane in response to an imposed osmotic gradient is much more rapid than the movement of the penetrant sugar molecules themselves (16), and thus any change in intracellular or extracellular osmotic activity results in a relatively rapid adjustment of cell volume. Here, the observed galvanometer deflection resulting from the addition of varying concentrations of nonpenetrant NaCl to volumes of dilute cell suspension was rectilinearly related to the change in the reciprocal of the osmotic activity as measured with the Fiske osmometer. Fig. 3 shows the result of one such experiment; the optical response of the cells to any imposed osmotic shift follows the pattern dictated (for the cell water volume) by the Boyle-van't Hoff relation. One may presume that the change in the galvanometer deflection with time similarly reflects the time course of volume changes resulting from sugar uptake in such dilute cell suspensions.

The heavy lines in Figs. 4 a and 4 b represent the observed uptake curves for D-galactose and D-fructose. Uptake curves predicted for simple passive diffusion for the same external sugar concentrations (represented by the light lines) were constructed from equation 3 a, the actual half-time of the uptake curve for the lowest concentration being used as the time unit. These
curves (represented by the light lines) are compared with the actual uptake data. The dashed portions of the experimental curves of both figures, having been obscured by the initial deflection due to shrinkage of the cells, are constructed by extrapolation to the initial galvanometer position at zero time (as predicted by the measured osmotic activity of the medium). The uptake pattern for D-fructose does not deviate markedly from the predictions for uncomplicated diffusion, whereas that of D-galactose is altogether out of line with this pattern. Fig. 4 a shows that, although the form of each curve singly does not differ greatly from that predicted by simple diffusion, the relation

![Graph](image-url)

Figure 3. The behavior of ascites tumor cells as osmometers. The experimental points illustrate the rectilinear relation between the galvanometer deflection in response to an osmotic disturbance (imposed by the addition of hyperosmotic concentrations of NaCl to a fixed volume of a dilute ascites cell suspension), and the reciprocal of the total isosmolality of the mixture as measured by the Fiske osmometer. The straight line is a best fit by eye through the points.

between the uptake curves at various penetrant concentrations cannot be accounted for on such a basis.

A direct comparison of the behavior of the various sugars studied is given by Fig. 2 b. Here the log \( t_{1/2} \) vs. \( C_o \) plots for several sugars are graphed on the same scale as the theoretical systems in Fig. 2 a. A spectrum of behavior is apparent; some sugars appear to follow satisfactorily the predictions for simple diffusion, while others deviate from these predictions to varying degrees (in one instance approaching the pattern of a completely saturated carrier model system).

Quantitative treatment of the results obtained from the densimetric experiments allows estimation of both a maximal velocity constant, \( k_o \), and an affinity constant, \( K \), for the various sugars tested. For this purpose, a graphi-
Figure 4 a. Densimetric records of D-galactose uptake by ascites tumor cells superimposed upon the theoretical uptake as predicted by uncomplicated diffusion. The uptake is expressed as a galvanometer deflection vs. time (heavy lines). The light lines represent theoretical uptake curves calculated from equation 1 c, based upon the half-times of the uppermost actual uptake curve. The dashed portions of the heavy lines represent extrapolation to the calculated galvanometer position at zero time. C<sub>0</sub> is, from top to bottom, 0.200, 0.410, and 0.805 isosmotic, respectively.

Figure 4 b. Densimetric records of D-fructose uptake by ascites tumor cells (heavy lines) superimposed upon the theoretical uptake (light lines) as predicted by simple diffusion. C<sub>0</sub> is, from top to bottom, 0.120, 0.296, and 0.772 isosmotic, respectively.
cal solution yields the best appreciation of the degree of uncertainty in the estimates. Thus from equation 3 b, each experimental value for $t_{1/2}$ at a particular $C_s$ defines a linear relation between $k_e$ and $K$. A solution for $k_e$ and $K$ is given by the intersection of any two such lines on the graph. Fig. 5 illustrates this type of analysis applied to half-time data from D-xylose and L-arabinose chemical uptake experiments. Although there is experimental uncertainty in the estimation of the half-times, Fig. 5 demonstrates a reasonable consistency in the location of the point of intersection given by the various pairs of curves for a given sugar. Table I lists the various sugars tested, and the $K$ and $k_e$ values assigned on the basis of the graphical solution of simultaneous equations. Inspection of the table shows that while $K$ varies from sugar to sugar, $k_e$ remains relatively constant.

**Figure 5.** Graphical solution for affinity and rate constants in the transport of D-xylose and L-arabinose into ascites tumor cells. Values of actual half-times of entry and the $C_s$'s for which the half-times were obtained were substituted into equation 3 b, thus defining $k_e$ as a function of $K$. The position of the intersection of a series of such functions for a given sugar thus defines the values of $K_e$ and $K$.

Chemical Analyses In the experiments involving direct chemical analytical methods, the cell sugar content was estimated as the difference between the total sugar in the cell plug and the amount of sugar calculated to be trapped in the interstices between the cells of the plug. The cell water volume in a given sample was determined by correcting the hematocrit (ascitocrit) cell volume for the solid cellular "dead space" (taken as 25% of the total cell volume at isotonicity). This average value was estimated by desiccating samples of cell suspension at 60°C for about a day, and weighing the residue. That equilibrium was reached between the cell water and external medium was demonstrated when sugar concentration calculated in the cell water
leveled off at essentially the same concentration as in the external medium. On a few occasions the sugar uptake ceased when the intracellular sugar concentration was still significantly lower than the extracellular concentration. Such preparations usually showed microscopic evidence of cytolysis. The failure of sugars to reach true equilibrium between the extracellular and intracellular water, reported by Luzzatto and Leonchini (24), might suggest some partial loss of integrity in their cell suspensions.

For estimation of the half-times of uptake, the data obtained by means of the chemical analytical method were plotted as cell sugar content against time (on a logarithmic scale), and a smooth line drawn through the points.

| Sugar        | $K$ (affinity) | $k_c$ (maximal velocity) |
|--------------|----------------|--------------------------|
| d-Glucose*   | 0.02           | 1.9                      |
| d-Xylose     | 0.05           | 3.0                      |
| d-Galactose  | 0.08           | 2.8                      |
| l-Arabinose  | 0.20           | 3.1                      |
| d-Arabinose  | 0.90           | 2.9                      |
| d-Ribose     | 0.90           | 3.2                      |
| l-Sorbose†   | >5             | —                        |
| l-Fructose‡  | >5             | —                        |
| l-Xylose‡    | >5             | —                        |

* d-Glucose, in high concentrations, is lethal to ascites tumor cells. Thus the values given are highly uncertain.
† l-Sorbose, l-fructose, and l-xylose appear to have such low affinities that no meaningful values can be assigned, (the intersections in the graphical solution falling in a region where the lines are nearly parallel, so that no satisfactory resolution is possible).

The same graphical solution of simultaneous equations as applied to the densimetric data was used to arrive at a maximal velocity constant, $k_c$, and affinity constant, $K$. These values are included in Table 1.

**Competitive Inhibition** An equivalent graphical method was used to estimate the constants in connection with the inhibitor experiments. The two sugars used here were d-xylose and l-arabinose. The half-times of uptake were determined experimentally by the chemical analytical method at several sugar concentrations, both in the presence and absence of the inhibitor. Theoretical log half-time curves (as a function of $C_o$) were constructed from equation 4 $b$ for various values of $K$ between 0.01 and 1.00 giving a spectrum of curves falling between the extremes shown in Fig. 2 $a$. The actual data plot ($t_{1/2}$ vs. $C_o$) for l-arabinose was drawn on tracing paper to the same
scales, and adjusted vertically over the theoretical curves to establish which
K value best approximated the data. This K agreed with that determined
by the graphical solution of simultaneous equations. Using this value for K,
similar log t₁/₂ vs. Cₒ curves were constructed from equation 4 b for the in-
hibited uptake, for various values of Kᵢ (at the Cᵢ used). Kᵢ could then be
estimated from the distribution of the data in this pattern of curves. Fig. 6 a
illustrates this procedure for inhibition of L-arabinose by diethylstilbestrol.
The Kᵢ appears to fall somewhere in the neighborhood of 2 × 10⁻⁵ M.

Similarly, a K value of 0.05 isosmolar was assigned for D-xylose; and the
corresponding theoretical curves constructed for xylose uptake in the presence
of diethylstilbestrol at the same concentration that was used in the L-arabi-
nose experiment. The results are given in Fig. 6 b; in this case, a value for
Kᵢ of 0.8 × 10⁻⁵ M fits well with most of the experimental points (the two
deviant points leaning toward the somewhat higher value given by the arab-
inose experiment).¹

In other less quantitative studies it was clear that both phloridzin and its
aglycone phloretin are fairly strong inhibitors of the sugar uptake.

Tracer Equilibrium The tracer uptake half-time was found to be slightly
less than 0.1 min for an external D-xylose concentration of 0.8 isotonic,
whereas the half-time for the net chemical sugar uptake in the same set of
experiments was just under 2 min. Because of delay in the sampling proce-
dure, half-times of less than 10 sec are subject to some uncertainty. Fig. 7
illustrates the (relatively short) extrapolation required in the log time vs.
cell tracer content curve to arrive at the estimate for the half-time of tracer
D-xylose entry. At this particular external sugar concentration, the ratio of
net chemical uptake half-time to tracer uptake half-time was about 20; the
ratio predicted by the theoretical model is 9.2.

DISCUSSION

Insofar as the present experiments overlap those of Crane, Field, and Cori
(14), the results are in reasonable mutual agreement. Crane et al. hesitated
to accept the possibility of a carrier mediation, since they felt that the very
high temperature coefficients they observed in the transport process were
indicative of a physiochemical membrane property instead of a chemical
reaction. We have noted this very high temperature coefficient; at 20°C the
rate of transport of most of the monosaccharides was nearly completely in-
hibited. However, the more explicit kinetic evidences are difficult to account
for other than by way of a carrier mediation. The demonstration of coun-
terflow-induced movement of monosaccharides through the ascites cell mem-

¹ In view of the poor aqueous solubility of the inhibitor (as previously noted), this order of discrep-
ancy in the apparent Kᵢ values calculated with the two sugars on different occasions is not taken
to be significant.
brane by Cirillo and Young (13) is strong evidence for mobile carrier involvement; and the demonstration in the present study of a rapid tracer uptake relative to gross chemical equilibration for a "high affinity" sugar is kinetically consonant with the predictions of the carrier model (5). The actual ratio
of the half-time of gross chemical equilibration to the half-time of tracer equilibration was not in exact accord with the independent estimate of the xylose $K$, as interpreted under the suggested model system: the predicted ratio for a $K$ value of 0.05 isosmotic and $C_0$ of 0.8 isosmotic is 9.2 (net/tracer), whereas the actual experimental value was, to the best approximation of the data, very close to 20. However, the simple diffusion equations predict an entirely different order of magnitude for this ratio: 1.2 (net/tracer). Thus the deviation in the data is in the opposite direction, and clearly excludes simple diffusion as the mechanism for the transmembrane movement of D-xylose.

![Graph](image)

**Figure 7.** Comparison of D-xylose uptake by ascites tumor cells with subsequent tracer xylose-14C after chemical equilibrium. Open circles, tracer uptake data as described in the text (technical). The half-time is smaller than the first measured incubation interval, and must therefore be arrived at by extrapolation. Closed circles, net chemical equilibration data. *Both lines are smooth curves drawn through the points.* The half-times are indicated by arrows on the time axis. The coordinates for net uptake are cell sugar content against time; for tracer uptake, net cell counts per minute against time. $C_0$ was 0.80 isosmotic.

With regard to mutual competition for uptake between pairs of monosaccharides, it is not at all surprising that Crane, Field, and Cori observed that D-xylose competed with L-sorbose for entry into tumor cells (at a low temperature), but that ribose did not compete with L-sorbose (at a higher temperature). There is little reason to ascribe any significance to the temperature difference, since one would expect the same behavior even if the temperatures were equal. The present study shows that both sorbose and ribose have such low affinities that they would not noticeably compete with one another for transport, while D-xylose has such a high affinity relative to sorbose that competition would be noticeable under any likely experimental circumstances.

The sequence of affinities of the sugars for the transport site given in Table
I is based on their comparative saturation behavior. Crane, Field, and Cori give a somewhat different sequence on the basis of estimates of first order rate constants. These were often rather haphazardly obtained; most of the incubation studies reported in their figures show only three points, and frequently only the single earliest point (at best two) fell sufficiently short of the final equilibrium level to be useful for defining the uptake rate. Thus, almost any value for half-time or initial velocity could be assigned, depending upon how one chose to extrapolate a curve back to the origin. Moreover, in many experiments (especially those conducted at 37°C), a first order rate constant could only be obtained after some rather arbitrary corrections of the data had been made.

The affinity sequence given in Table I closely parallels that established in similar studies on the human erythrocyte (3, 4), in which the affinity constants correlate well with the relative conformational stabilities of the sugar; i.e., those molecules tending to be most stable in the Cl “chair” conformation (as designated by Reeves (25)) are the preferred substrates for this transport process. In a few experiments conducted with D-glucose it was evident that saturation developed at a lower concentration than with any other sugar studied, but since glucose is metabolized by ascites tumor cells, these studies were not quantitative (Table I). D-Xylose, the pentose homomorph of D-glucose, is not metabolized by the tumor cells, has a highly stable Cl conformation, and has a high measured affinity for the transport system. Its mirror image enantiomorph, L-xylose, correspondingly stable in the 1C conformation, has an unmeasurably low affinity. The two enantiomorphs of arabinose fall in the reverse order, in an intermediate position, in line with their stability ratings in the preferred conformation. In these respects the tumor cell behaves similarly to the human erythrocyte. The effects of particular configurational changes studied by Crane, Field, and Cori in relation to the “affinities” (as measured by the first order rate constants) fit into this pattern. Special emphasis was placed by Crane, Field, and Cori on the necessity for a glucose-like configuration at the number 2 and number 3 carbon atoms. However, it was noted that 3-methyl glucose retained a high “affinity,” while other configurational changes at the number 3 carbon atom of glucose produced lowered affinities. This situation is understandable in view of the fact that the 3-methyl substitution (in the equatorial position) does not interfere with the stability in the Cl conformation, whereas the other configurational changes mentioned refer to sugars (D-allose, D-ribose, and D-arabinose) having in the Cl conformation an axial hydroxyl group (an unstabilizing influence) at the 3-position.

Collectively, the data indicate that, although $K$ varies greatly from sugar to sugar, $k_c$ is relatively invariant. This behavior would be anticipated (see 3 The values assigned for $K$ and $k$ using data from both methods were not significantly different.)
below) if the rate-limiting factor in the transport were the diffusion of a sugar-carrier complex through the membrane (assuming that the attachment of any sugar to the carrier would result in a complex of similar mobility in the membrane substance, because the bulk and physicochemical attributes added to the carrier would be approximately the same for any monosaccharide). The fact that $k_c$ appears to be constant while $K$ varies strengthens the argument for the postulates of the model system.

With respect to the inhibitor studies, the data support the conclusion that the action of diethylstilbestrol is based on a simple competition with the monosaccharides for a binding site on the membrane surface. The resultant theoretical predictions for the relative degrees of inhibition of sugar uptake exerted by stilbestrol with sugars of differing affinities and at varying concentrations are reasonably consonant with the experimental data. The qualitative observation that phloridzin is also effective in inhibiting sugar transport in the ascites cell, confirms the finding of Crane, Field, and Cori (14), but is the only noted feature distinguishing this transport from that in the erythrocyte. In the latter cell, the aglycone phloretin is a potent transport inhibitor, but phloridzin is only very weakly active. However, the apparent $K_f$ value for diethylstilbestrol was nearly the same for both systems, on the order of $10^{-3}$ M for the ascites cell, and approximately $0.2 \times 10^{-4}$ M for the erythrocyte.

There is evidence for carrier involvement in the transport of other substances, notably amino acids (27), across the ascites tumor cell membrane. However, to the present time all such evidence (including that presented here) essentially is in the nature of kinetic (mathematical) inference. While most investigators agree that this evidence logically demands postulation of a carrier entity, any definitive conclusion must await the extraction and characterization of the membrane element responsible for the transport phenomena; and this should be the imperative consideration in planning future research in this field.

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3 This difference becomes insignificant because diethylstilbestrol, at the concentration used, is strongly bound to the cells, having a cell to medium ratio of about 90(26). The $K_f$ value determined for the action of stilbestrol on the erythrocyte was based upon experiments in which the cells constituted only about 0.3% of the solution, whereas, in these experiments, the cells occupied about 20% of the total volume of solution. Thus, the effective concentration of inhibitor in the medium was considerably lower than $1.66 \times 10^{-4}$ M, and consequently, the value of $K_f$ is actually somewhat lower than that given here.
A thesis covering this study was also submitted to the University of Louisville by Alan R. Kolber in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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