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Research Article

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Transport of Monosaccharides

I. ASYMMETRY IN THE HUMAN ERYTHROCYTE MECHANISM

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ABSTRACT Transport of D-glucose across human erythrocyte membranes occurs via a facilitated diffusion process which demonstrates influx-efflux asymmetry. The mechanism of the asymmetry has been studied by estimating unidirectional fluxes in the presence or absence of trans equilibrium hexose. In the absence of trans hexose, the half-saturation constant for efflux at 15°C was approximately 10 mM as compared with 27 mM for influx; the corresponding values for maximal transfer rates ($\mu\text{mol}/\text{min}$ per ml cell H_2O) were approximately 51 vs. 18. The estimation of kinetic parameters, including the constant F_s , which is the ratio of maximal transfer rate/half-saturation constant, indicates a unique effect of intracellular hexose on the transfer system. Further evidence to support this conclusion was obtained by studying the effects of noncompetitive inhibitors on efflux vs. influx. *N*-ethylmaleimide, *p*-chloromercuribenzenesulfonate, and dichloroallyldiethylstilbestrol all inhibited efflux much more than influx. Glucose rendered the transport system more reactive to *N*-ethylmaleimide as assayed by efflux, whereas influx was much less affected. The results support the hypothesis that the transport system exists in two states. Transition from one state to the other is dependent on the presence of intracellular hexose.

INTRODUCTION

It is well established that D-glucose and related monosaccharides are transferred across human erythrocyte membranes by facilitated diffusion, a mechanism which equilibrates the extra- and intracellular concentrations of sugar (1-4). At equilibrium the rate of influx equals that of efflux. Facilitated diffusion mechanisms can be categorized further with respect to influx vs. efflux as *symmetric* or *asymmetric*. In a symmetric mechanism the influx rate equals the efflux rate when the fluxes are esti-

mated separately with the same concentration of sugar on the appropriate (cis) side of the membrane and either no sugar on the opposite (trans) side or similar concentration gradients cis/trans. In contrast, an asymmetric mechanism results in unequal rates of influx as compared to efflux under the foregoing conditions. It bears emphasis that the steady state reached with both asymmetric and symmetric mechanisms is an equilibrium at which time the unidirectional fluxes are equal.

In a prior report (5) we noted that according to the foregoing criteria D-glucose transport across human erythrocyte membranes is an asymmetric facilitated diffusion, and the present studies were undertaken to define the mechanism of the asymmetry. Inasmuch as the unidirectional flux rates follow Michaelis-Menten kinetics (1-4), one could ascribe the observed influx-efflux inequalities to differences in (a) the half-saturation constants (K_t),¹ (b) the rates of maximal flux (V_m), or (c) both. As demonstrated in the Appendix, different K_t values for influx as compared to efflux could result from different rate constants for sugar-carrier interactions at the opposite surfaces of the erythrocyte membrane;² un-

¹ Abbreviations used in this paper: B_s , half-saturation constant for flux into trans equilibrium glucose; C_t , total carrier concentration at interfaces; DCDS, 3,3'-di-2-chloroallyldiethylstilbestrol; F_s , ratio of maximal transfer rate to half-saturation constant; K_t , half-saturation constant for flux in absence of trans glucose; NEM, *N*-ethylmaleimide; PMBS, *p*-chloromercuribenzenesulfonate; S_i , S_o , intracellular and extracellular sugar concentrations, respectively; V_o , efflux rate; V_i , influx rate; V_m , rate of maximal flux.

² The term "carrier" is used in this report to designate a membrane constituent which brings about a specific transfer process by binding or associating with a particular transported species. We use the term "mobile-carrier" to designate the specific mechanism illustrated in Fig. 5. These definitions are necessary because two recent models (8, 9) did propose specific membrane entities which bind or associate with the transferred species, yet they were designated "noncarrier" models. By our definition the term for these last models would be "nonmobile-carrier" models.

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equal V_m values could signify differences in the number of functional carriers involved in the respective fluxes. The results of a systematic evaluation of the constants and the effects of a number of inhibitors of the transport are described below. They indicate that the influx-efflux asymmetry results from a change in the carrier system owing to the presence of intracellular hexose which alters all the kinetic parameters and the reactivity of the transport system toward diverse inhibitors. The observations support the existence of two carrier states, rather than a single carrier mechanism, in the erythrocyte membrane.

METHODS

Working hypothesis. The general treatment described by Regen and Morgan (6) of the mobile carrier model was extended as shown in the Appendix to provide the initial theoretical framework for these studies. The rates of influx or efflux of D-[¹⁴C]glucose in the presence or absence of equilibrium trans D-glucose were estimated as functions of the cis sugar concentration. Equations 7-10 in the Appendix are the flux equations and constants which apply. The constants were evaluated by suitable plots. Table I summarizes the relationships derived in the Appendix between the flux equation constants and the ratios of individual rate constants of the carrier cycle illustrated in Fig. 5. Although the applicability of a *symmetrical* mobile carrier model has been questioned (7-9), the unrestricted model (6) proved quite useful as a framework for these studies, particularly as our initial observations indicated an asymmetric mechanism.

Procedures. Human erythrocytes were obtained from blood bank specimens preserved with ACD solution (each 250 ml blood contained 0.3 g citric acid, 0.825 g sodium citrate, and 0.918 g dextrose) at 5°C for 3-7 weeks prior to use. Fresh blood gave essentially the same results, as noted below. The erythrocytes were obtained by centrifugation at 3,000 rpm for 10 min at 5°C in an International centrifuge (International Scientific Instruments Inc., Palo Alto, Calif.) and washed twice with 9 vol of "washing buffer" (30 mM sodium phosphate of pH 7.4 containing 117 mM NaCl and 2.8 mM KCl) prewarmed to 37°C. After each suspension in buffer the cells were shaken at 37° for 5 min before centrifugation. Estimations with the glucostat method (10) showed that the procedure decreased the intracellular glucose from an initial value of approximately 14 mM to 0.1 mM. Inasmuch as comparisons of influx and efflux were sought, the procedures were designed to treat the cells comparably prior to flux estimations. Thus all cells were loaded initially by shaking at 37°C for 2 h in "loading buffer" (26 mM sodium phosphate of pH 7.4 containing 122 mM NaCl and 2.5 mM KCl) containing an appropriate concentration of D-glucose and, for efflux estimations only, 0.8 μCi/ml of uniformly labeled D-[¹⁴C]glucose (New England Nuclear Corp., Boston, Mass.; specific radioactivity 1-5 mCi/mmol). Subsequent treatment of the loaded cells varied with each particular estimation as follows.

Influx, no trans glucose. Cells were washed three times at 37°C with 9 vol of glucose-free loading buffer using the wash procedure described above. In the first wash alone the intracellular glucose concentration was balanced by addition of an isosmotic quantity of NaCl to the buffer. Following the third wash and centrifugation, as much of

TABLE I
Relationships Deduced from the General
Mobile-Carrier Model (Appendix)*

Ratio of constants	Relationships deduced
K_{to}/K_{ti}	If $K_{to}/K_{ti} = 1$, $k_{-1}/k_3 = 1$ † If $K_{to}/K_{ti} > 1$, $k_{-1}/k_3 < K_{ti}/K_{to}$
B_s/K_{ti}	If $B_s/K_{ti} \geq 1$, $k_2/k_4 > 2 B_s/K_{ti}-1$ If $B_s/K_{ti} \geq 1$, $k_3/k_4 > 2 B_s/K_{ti}$
B_s/K_{to}	If $B_s/K_{to} \geq 1$, $k_{-1}/k_4 > 2 B_s/K_{to}$

Fig. 5 indicates the forward and reverse reactions characterized by k_1 to k_4 . In a facilitated-diffusion mechanism symmetrical with respect to influx and efflux, $K_{to} = K_{ti}$, $k_{-1} = k_3$ and $k_1 = k_3$.

* B_s , K_{ti} , and K_{to} are defined in the Appendix.

† As shown in the Appendix $k_{-1}/k_3 = k_1/k_3$.

the supernatant fluid as possible was aspirated, and the packed cell suspension was equilibrated at 15°C for at least 10 min and then dispensed for flux estimations via disposable plastic pipets (Falcon Plastics Div., Oxnard, Calif.; 1-ml size). These pipets have suitably wide tips and were shown to deliver aliquots of 0.20 ml of packed cell suspension (hematocrit values ranging 0.81-0.98) with better than 97% reproducibility when the meniscus was drawn up to the 0.25-ml mark. Each flux was estimated in duplicate at 15°C as the difference between an initial (zero-time) and a final (usually 15-30 s) timed sample. To initiate influx 0.20 ml of packed cells were added to 0.75 ml of "test buffer" (final composition: 9 mM sodium phosphate, pH 7.4; 0.8 mM KCl; appropriate D-glucose plus NaCl to yield 325 mosm/liter; and 1-3 μCi/ml of D-[¹⁴C]glucose), the suspension was shaken at 15°C, and the transport was terminated by addition of 7 ml of ice-cold "stopping solution", 2 mM HgCl₂-310 mM NaCl (11). For the zero-time samples the stopping solution was premixed with test buffer and the cells were then added. After rapid mixing the cells were quickly and efficiently separated from the ambient medium by centrifugation through liquid silicone (12). For the separation 1.0 ml of 3 M perchloric acid was placed carefully on the bottom of a 2½ × ½ in cellulose nitrate centrifuge tube (Beckman Instruments, Palo Alto, Calif.) and 3 ml of liquid silicone (Versilube F 50, density 1.05, Harwick, Inc., Trenton, N. J.) layered on top. The tube was kept in ice until use when 1.0 ml of the ice-cold erythrocyte suspension was layered over the silicone and the whole centrifuged for 2 min at 3,000 rpm and 5°C in the swinging bucket head of an International centrifuge. After centrifugation the top-most layer (extracellular medium) and the bottom perchloric acid layer containing soluble substances leached from the cells were collected. The latter fluid was obtained by puncturing the tubes with a fine needle inserted just above the flat pellet of denatured cell proteins. To estimate [¹⁴C]glucose the perchloric acid extracts were neutralized with NaOH and counted in Bray's solution (13), using a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Sodium perchlorate equal to that formed in the foregoing samples was routinely added to counting vials containing the extracellular and other fluids to be assayed. The

influx rate in units of micromoles glucose/minute per milliliter cell water was calculated as the increment in intracellular radioactivity divided by the specific radioactivity of the [^{14}C]glucose of the test solution. The volume of cell water was obtained in each estimation from the hematocrit, using a correction for trapped extracellular water. As estimated with [^{14}C]inulin (New England Nuclear Corp., specific radioactivity 7.7 mCi/g) trapped extracellular water accounted for an average of 19% of the packed cell volume (range 11–31% in determinations with six different cell lots) in the dense suspensions used. The volume of cell water was assumed to be 75% of the cell volume.

The following variations of the procedure did not influence significantly the results obtained: dilution of cell suspensions to hematocrit values of approximately 50% prior to mixing with test buffer; modification of the test buffer to contain 23 mM sodium phosphate, pH 7.4 and 2.1 mM KCl to make the composition identical to that used in the efflux studies below.

Efflux, no trans glucose. Cells loaded with D-[^{14}C]glucose were cooled to 5°C and maintained at this temperature through two washes with chilled, glucose-free loading buffer modified to contain NaCl in amounts sufficient to balance osmotically the intracellular glucose concentrations. After the final centrifugation the supernatant fluid was removed as completely as possible and the packed cell layer was equilibrated at 15°C for at least 10 min. Flux estimations were performed in duplicate, at 15°C, as described for influx. Aliquots of 0.20 ml of cells were transferred to 0.75 ml of glucose-free test buffer (same composition as the buffer used to wash the loaded cells) and the transfers terminated by addition of stopping solution. Zero-time samples were obtained by adding the cells to premixed stopping solution plus test buffer. Liquid silicone was employed to separate cells from their ambient medium, and the efflux rate ($\mu\text{mol}/\text{min}$ per ml cell water) was calculated from the decrease in intracellular D-[^{14}C]glucose, determined by assay of the perchloric acid extracts, and the specific radioactivity of the glucose used to load the cells.

Two variations in the standard procedure were tested and did not alter significantly the results discussed below. In four experiments cells which had been loaded with [^{14}C]glucose, chilled, and washed were not equilibrated to 15°C before the flux assay in order to minimize the concentration of extracellular glucose present during the flux estimation. In two additional experiments the washing procedure was made more comparable to that used for influx assays. The cells were first washed five times with 9 vol of buffer exactly as described for the influx method. Subsequently, they were loaded with [^{14}C]glucose and centrifuged, the supernatant fluid was aspirated, and samples of the packed cells were transferred for the flux assay.

Fluxes, trans glucose. Influx and efflux in the presence of trans equilibrium glucose were estimated by a double isotope procedure. Erythrocytes were loaded with [^{14}C]glucose at various concentrations of nonradioactive glucose as described above. The cells were packed by centrifugation, the supernatant solutions removed as completely as possible, the cells were equilibrated for 10 min at 15°C and 0.20-ml aliquots of the cell layer were transferred to 0.75 ml test buffer prepared to contain the appropriate equilibrium concentration of nonradioactive glucose plus 2.7 $\mu\text{Ci}/\text{ml}$ of D-[^3H]glucose (New England Nuclear Corp., specific radioactivity 3.85 mCi/mg, labeled in the C2 position). Fluxes were assayed at 15°C, terminated with stopping solution, and cells were separated by centrifugation through liquid silicone. Zero-time samples were obtained as previously de-

scribed. The isotope concentrations in the perchloric acid extracts were estimated by liquid scintillation spectrometry, and the flux in each direction was calculated as described above from the changes in intracellular concentrations and the known specific radioactivities.

Inhibitor studies. The effects of the reversible inhibitors *p*-chloromercuribenzenesulfonate (PMBS) and 3,3'-di-2-chloroallyldiethylstilbestrol (DCDS)^{*} were tested as follows. For efflux, erythrocytes were washed five times, loaded at a hematocrit of 33% with [^{14}C]glucose for 1 h at 37°C, and portions of the loaded suspension were treated with the appropriate compound for an additional 5 min at 37°C. The cells were then centrifuged and aliquots of the packed cell layer taken for flux estimation in a test buffer containing the appropriate concentration of the inhibitor and no glucose. For influx, the procedure described above for estimating influx, no trans glucose, was followed except that the loading period with glucose was shortened to 1 h and the cells were washed three times. After the third resuspension and centrifugation, sufficient supernatant fluid was removed to permit resuspension to a hematocrit of 33%, and the cells were treated with the inhibitor for 5 min at 37°C. Finally, the suspensions were centrifuged and the cells assayed in test buffer containing nonradioactive and [^{14}C]glucose along with the appropriate inhibitor concentration.

N-ethylmaleimide (NEM), an irreversible sulfhydryl inhibitor, was tested as described above with the following modifications. For both influx and efflux cells either loaded with glucose or unloaded were treated with 10 mM NEM at 37°C, and the reaction was terminated after 11–22 min by adding a 1.5-fold excess of mercaptoethanol. Cells for efflux assay were packed by centrifugation and tested as described above in test buffer minus NEM. Cells for influx estimations were washed three times to remove intracellular glucose, packed by centrifugation, and assayed in test buffer minus NEM.

Errors and corrections. In estimating unidirectional fluxes without trans glucose a finite concentration of the hexose builds up on the trans side of the membrane and results in error owing to (a) trans effects such as counter-transport and (b) backflux. The present studies were designed to minimize such errors by (1) estimating the fluxes under conditions of minimal trans/cis glucose ratios; (2) confirming the basic asymmetry of influx/efflux directly via estimations in the presence of similar trans/cis ratios (see Table III below); and (3) calculating the backfluxes and assessing the effects of such corrections on the values of the kinetic constants.

Fig. 1 illustrates the average values for cis and trans glucose during the short flux periods in the experiments performed initially. (The average values were within 10% of the mean values and the former were therefore used routinely.) For influx assays the average trans/cis ratio was approximately 5% and for efflux approximately 11%. The small ratio for influx assays is especially noteworthy since influx involved transfer from an extracellular compartment approximately six times the volume of the intracellular compartment.

The influx rates were also linear (see Fig. 2) in the short flux periods employed. The small trans/cis ratios suggest that the present data under conditions of "no trans

^{*} DCDS is highly insoluble in water and was dissolved in ethanol. When added to erythrocyte suspensions the final ethanol concentration remained less than 1.0%, and control suspensions contained identical ethanol concentrations.

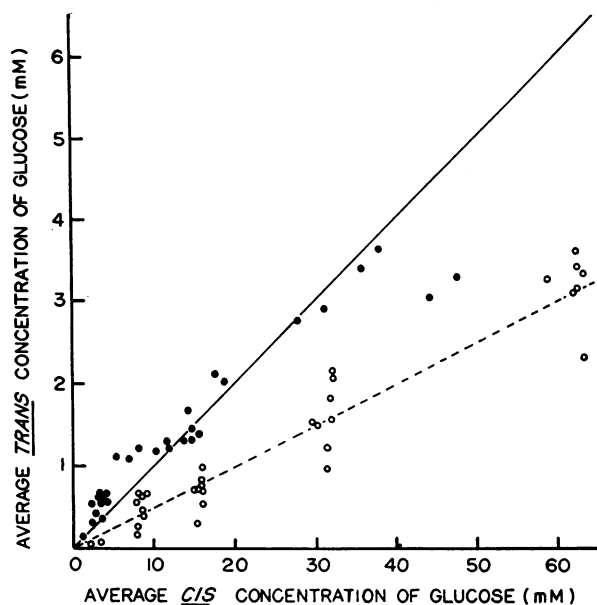


FIGURE 1 Average trans and cis concentrations of glucose during periods of efflux (●) or influx (○) in the present studies. The upper line represents a trans/cis ratio of 10% and the lower, broken line a trans/cis ratio of 5%.

glucose" provide a reasonable approximation of the true K_t values, i.e., the half-saturation constants defined in the Appendix for trans/cis ratios equal to zero. Moreover, the difference in trans/cis ratios between the efflux and influx assays cannot account for the influx/efflux asymmetry of the transport described below. The major factor in the asymmetry is a relatively high F_s (and V_m) for efflux as compared with influx. However, the value of the F_s constant estimated for efflux is not significantly affected by the presence or absence of trans equilibrium glucose (Table

III). In addition, the estimated K_t for efflux was less than that for influx, although the higher trans/cis ratios for efflux should increase the K_t value observed. Table II, for example, shows that the half-saturation constant for efflux into trans equilibrium glucose (B_s) is four to six times greater than that in the absence of trans glucose (K_t). The difference in K_t for influx versus efflux is thus underestimated in these studies.

Direct experimental evidence to confirm the influx/efflux asymmetry was obtained in additional studies in which efflux and influx were estimated in the presence of similar trans/cis ratios (Table III) ranging 3.2–6.5%. Efflux assays were performed with a packed cell volume of 7.5%, as compared with 25% in the foregoing experiments, to decrease trans sugar.

For the "no trans sugar" observations, errors owing to the finite trans/cis ratios could be approximated and the unidirectional flux rates corrected by calculating the back fluxes according to the general equations 1 and 2 for influx (V_i) and efflux (V_e) in the Appendix. The observed flux, V_{net} , equals $V_i - V_e$. When corrections were calculated using the constants derived from the raw data, the major constant determining the backflux values was found to be F_s , and the value of F_s itself was relatively unaffected by the corrections (Table II). Further, the influx/efflux asymmetry in K_t was apparent whether or not the corrections were used. Unless specified, flux values below for "no trans" observations are not corrected for backflux.

Backflux errors were more significant in assays with equilibrium trans glucose because of the very rapid transfers. Thus a correction for backflux was made routinely using the relationship: flux (cis \rightarrow trans) = change in cis compartment radioactive glucose / (average specific radioactivity in cis compartment minus average specific radioactivity in trans compartment).

To determine the extent to which ice-cold stopping solution effectively terminated all further transfer the following experiment was performed. Washed erythrocytes were loaded with [14 C]glucose, and at equilibrium portions were centrifuged to permit direct estimation of glucose in the extra- and intracellular compartments. Aliquots of the

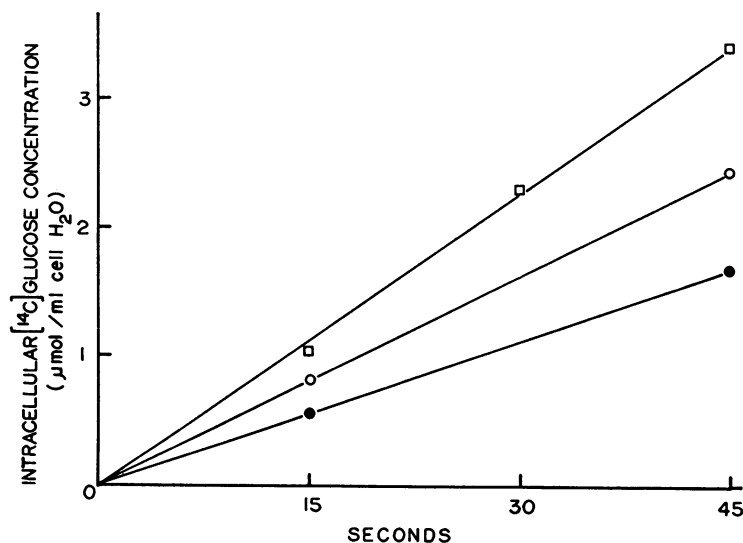


FIGURE 2 Linear influx of [14 C]glucose determined in the absence of trans glucose and using cis concentrations of 13 (●), 26 (○), and 53 (□) mM hexose.

packed cells were then rapidly diluted into ice-cold stopping solution plus test buffer, as described previously for efflux assays at zero time, and the samples immediately centrifuged through liquid silicone. The [^{14}C]glucose content of the top, extracellular phase was estimated and compared with the ^{14}C carried over in the extracellular water of the packed-cell aliquots prior to dilution. In two experiments 7.0 and 13.7% (mean: 11%) of the intracellular [^{14}C]glucose leaked out of the cells after mixing with the stopping solution. Thus in efflux assays we routinely corrected for an outward leak of 11%. The correction was negligible in influx assays, for in contrast to efflux, after addition of stopping solution the ratio of [^{14}C]glucose extracellular/intracellular was close to unity and the leak did not affect the values for cis glucose concentrations.

Finally, it is noteworthy that flux values were always estimated as differences between zero-time and later samples. Thus systematic errors arising from the foregoing leak or from the small quantity of extracellular fluid carried by the cells through the liquid silicone (12) were compensated.

RESULTS

Linear kinetics of influx. Despite the relatively large literature concerning glucose efflux from erythrocytes, systematic, well-controlled data comparing efflux and influx are not available, and influx in general has not been as well studied. The liquid silicone method described here provides a useful and convenient procedure for estimating influx over periods as short as 10–15 s. Fig. 2 illustrates the linear uptake of glucose observed over a period of 45 s by cells exposed to either 13, 26, or 53 mM glucose.

Comparison of influx and efflux. Nine different blood samples were each tested for influx and efflux in the ab-

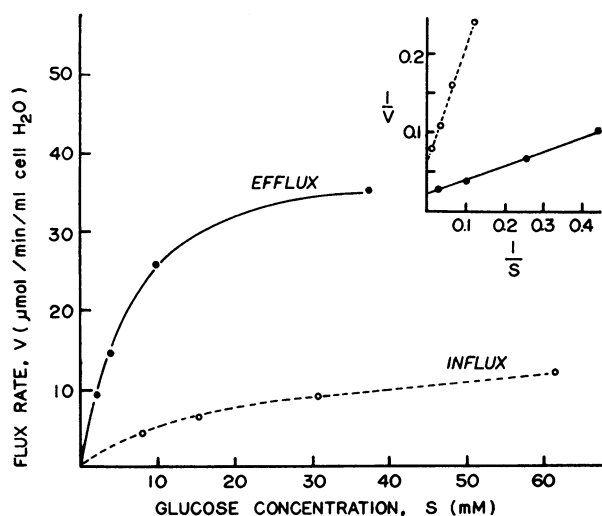


FIGURE 3 Relationship of efflux (●—●) or influx (○---○) rate to cis glucose concentration in the absence of added trans glucose. Inset shows the same data plotted according to the method of Lineweaver and Burk (14). All values have been corrected for backflux as discussed in Methods.

sence of trans glucose and at four cis concentrations of the hexose. The mean flux rates observed were plotted as functions of the cis concentrations in Fig. 3, and they demonstrate typical Michaelis-Menten kinetics. Throughout the range tested efflux considerably exceeded influx at comparable cis concentrations. When plotted by the double-reciprocal method of Lineweaver and Burk (14), typical linear plots were obtained (Fig. 3, inset). Table II summarizes the values obtained for the kinetic constants defined in equations 7–10 of the Appendix. Values for data uncorrected and corrected for backflux are listed. Both sets of values indicate that the mean half-saturation constant for influx, K_{i0} , considerably exceeds that for efflux, K_{e0} ($P < 0.01$). The mean K_{i0}/K_{e0} ratios were 1.9 and 2.6, respectively, for uncorrected and corrected data. Even more striking was the relatively higher value for the F_s constant for efflux as compared with influx ($P < 0.001$), with efflux/influx ratios of 5.0 and 7.4, respectively. Inasmuch as V_m , the maximal transport rate, is the product of F_s and K_i (Appendix, equations 11 and 12), V_m for efflux considerably exceeds that for influx ($P < 0.001$) owing to the higher F_s value; K_{i0} is less than K_{e0} and this factor minimizes the difference in maximal transport rate between the fluxes. The values in Table II for K_i of efflux (15°C) are 6.6 ± 1.1 mM (uncorrected) or 10.3 ± 4.6 mM (corrected), in fair agreement with recently published values of Miller (15), 7.4 ± 1.4 mM (20°C), and Karlish, Lieb, Ram, and Stein (16), 25 ± 3 mM (20°C).

Table II also lists the values observed for the kinetic constants for influx and efflux into equilibrium trans glucose. The results of experiments with four different blood samples tested by the double isotope method are summarized. Under equilibrium conditions influx and efflux do not differ significantly with respect to V_m , F_s , or the half-saturation constants (B_s). The ratios of B_s/K_i considerably exceed one, with values of 4.0 and 1.9 for efflux and influx, respectively. Values for efflux in excess of one have been reported previously (17, 18) and interpreted in terms of mobility of loaded vs. unloaded carriers (see Discussion).

A striking and unexpected observation concerning the F_s constant emerges from inspection of Table II. The mean values of F_s are 5–6 when flux in either direction is estimated in the presence of *intracellular hexose*, i.e., for efflux with and without equilibrium trans glucose, and for influx with trans glucose. In the absence of intracellular glucose, the condition for testing influx without trans sugar, the F_s constant is approximately 1.0. Intracellular glucose, a necessary condition for efflux estimations, thus seems to influence the transport mechanism itself. The significance of these observations is discussed below.

TABLE II
Kinetic Parameters of D-glucose Transport Across Human Erythrocyte Membranes (15°C)*

Flux	Kinetic parameter		
	F_s	K_t or B_s	V_m
	ml/min/ ml cell H ₂ O	mM	μmol/min/ ml cell H ₂ O
Efflux, no trans sugar	5.0±1.6	6.6±1.1	31.2±9.3
Influx, no trans sugar	1.0±0.4	12.2±4.8	9.3±3.0
<i>P</i>	<0.001	<0.01	<0.001
Efflux, no trans sugar, backflux correction	5.9±2.5	10.3±4.6	50.6±19.5
Influx, no trans sugar, backflux correction	0.8±0.5	26.9±12.5	17.9±6.4
<i>P</i>	<0.001	<0.01	<0.001
Efflux, trans equil. sugar	6.2±1.5	41±17	232±64
Influx, trans equil. sugar	4.9±0.7	50±6.0	231±33

* The kinetic constants are defined in the Appendix. K_t refers to "no trans" experiments (nine in number) and B_s to trans equilibrium sugar experiments (four in number). Values are given as means±SD. *P* values are for the influx-efflux differences. In the trans equilibrium sugar experiments the differences were not significant. The backflux correction is described in Methods.

As discussed in Methods, the values for "no trans" fluxes in Table II were obtained with average trans/cis ratios of 5% and 11% for influx and efflux, respectively. Hence further experiments were designed to estimate the fluxes in the presence of equal trans/cis ratios and the results are summarized in Table III. The cis concentrations were chosen to yield maximal flux rates and the mean trans/cis ratios for influx and efflux, respectively,

were 4.7% (range 3.2–6.5%) and 4.8% (range 3.8–6.1%). In each of four experiments the efflux rate exceeded the influx rate (t [paired differences] = 12.8, $P < 0.01$) and the efflux/influx ratio ranged 3.4–5.7. The corresponding mean value for the ratio of efflux/influx in the preceding observations (Table II) is 3.0. These observations provide direct experimental verification of the influx/efflux asymmetry of the transfer mechanism.

TABLE III
Flux Ratios (Efflux/Influx) with Various Trans/Cis Glucose Ratios*

Exp. no.	Direction of flux	Cis glucose conc.	Average trans/cis glucose ratio	Flux rate	Flux ratio, efflux/influx
		mM		μmol/min/ml	
1	Efflux	46.7	0.048	19.3	3.6
	Influx	62.5	0.043	5.3	
2	Efflux	33.0	0.061	20.8	3.7
	Influx	61.7	0.046	5.6	
3	Efflux	48.9	0.038	22.8	5.7
	Influx	62.5	0.032	4.0	
4	Efflux	40.9	0.044	27.4	3.4
	Influx	62.5	0.065	8.1	
Grouped data†	Efflux	37.5±7.6	0.110	19.3±4.8	3.0
	Influx	61.7±1.8	0.050	6.5±0.7	

* Experiments 1 and 2 were performed with one blood sample and 3 and 4 with another. Cis glucose concentrations were chosen to yield maximal flux rates.

† Grouped data represent seven determinations with seven different samples; means±standard deviations are shown.

They also indicate that the efflux rates observed above with a mean trans/cis ratio of 11%, 19.3 ± 4.8 $\mu\text{mol}/\text{min}$ per ml cell H_2O , do not differ significantly from the rates observed with a mean trans/cis ratio of 4.8%, 19.3–27.4 (mean 22.6) $\mu\text{mol}/\text{min}$ per ml.

To verify the observed asymmetry of the human transfer mechanism and to validate further the methods used, flux studies were also performed with rabbit erythrocyte suspensions containing a high proportion of reticulocytes. Regen and Morgan (6) have reported that the rabbit erythrocyte mechanism is symmetrical. Accordingly, cell suspensions from each of three rabbits treated with phenylhydrazine were tested for influx and efflux of D-glucose as described in Methods. The cis concentrations for influx and efflux, respectively, were 8.2 and 8.1 mM, and the corresponding flux rates were 0.36 (range 0.28–0.45) and 0.43 (range 0.19–0.62) $\mu\text{mol}/\text{min}\cdot\text{ml}$ cell H_2O , not significantly different. Thus the results confirm the symmetry of the rabbit transfer as compared with the marked asymmetry of the human mechanism.

Comparisons of [^{14}C]glucose and glucostat methods. Particularly in view of the findings above, it was desirable to compare the kinetic constants estimated by means of [^{14}C]glucose with values obtained by specific chemical estimation of glucose via the glucostat method (10). The flux procedures in the absence of trans glucose, as described above, were modified as follows. After addition of cold stopping solution to terminate the flux, 4 ml of the resulting mixture were layered over 3 ml of liquid silicone layered in turn over 1 ml of 1.5 M K_2CO_3 in a $3 \times \frac{5}{8}$ in cellulose nitrate tube (Beckman Instruments, Inc., Fullerton, Calif.). The tube was centrifuged, and all fluid above the K_2CO_3 (bottom) layer removed and the walls wiped clean. Sodium dodecyl sulfate and ethylenediamine tetraacetate were added to final concen-

TABLE IV
Kinetic Parameters of D-glucose Transport Determined by Tracer and by Chemical Estimation*

Flux	Method	Kinetic parameter		
		F_s	K_t	V_m
		$\text{ml}/\text{min}/$ $\text{ml cell H}_2\text{O}$	mM	$\mu\text{mol}/\text{min}/$ $\text{ml cell H}_2\text{O}$
Efflux	[^{14}C]glucose	5.3	7.5	39.1
	Glucostat	7.2	5.1	37.1
Influx	[^{14}C]glucose	1.0	15.8	7.0
	Glucostat	0.6	17.0	10.0

* Mean values of three experiments with separate blood samples are listed. Fluxes were estimated in the absence of trans glucose. Differences between the tracer and glucostat methods were not statistically significant.

TABLE V
Kinetic Parameters of 3-O-methylglucose Transport Across Human Erythrocyte Membranes (15°C)*

Exp.	Flux	Kinetic parameter		
		F_s	K_t	V_m
		$\text{ml}/\text{min}/$ $\text{ml cell H}_2\text{O}$	mM	$\mu\text{mol}/\text{min}/$ $\text{ml cell H}_2\text{O}$
1	Efflux	8.3	2.4	20.0
	Influx	1.0	5.0	5.0
2	Efflux	10.0	1.6	16.0
	Influx	1.0	6.1	6.1

* Observations with two separate blood specimens. Fluxes were estimated without trans hexose, using D-[3-O-methyl- ^{14}C]glucose (New England Nuclear Corp., 95 mCi/g). For efflux and influx the cells were washed identically, i.e., five times with 9 vol of wash buffer at 37°C, with shaking for 5 min during each wash.

trations of 0.1% and 2 mM, respectively, and the erythrocytes lysed by shaking at room temperature for 15 min. The lysate was chilled in ice, half-neutralized with perchloric acid and the precipitate removed by centrifugation. The supernatant fluid was deproteinized with $\text{Ba}(\text{OH})_2$ and ZnSO_4 (19), the precipitate spun off, and the resulting supernatant fluid adjusted to pH 7 with KH_2PO_4 . Separate aliquots were then either assayed with the glucostat reagents or counted in Bray's solution. This procedure was necessary to ensure quantitative recoveries of glucose and to avoid inhibition of the glucostat enzyme by the mercury of the stopping solution.

The results of simultaneous estimations using three different blood samples are shown in Table IV. The mean values of the kinetic constants obtained with the two methods are in good general agreement and confirm the prior observations. Efflux F_s and V_m considerably exceed the influx values, whereas the reverse holds for the K_t constants.

Studies with 3-O-methylglucose. To determine whether the foregoing influx-efflux differences result from metabolic effects of D-glucose, flux studies in the absence of trans sugar were undertaken with the nonmetabolized hexose 3-O-methylglucose. The results with two separate blood samples are listed in Table V and demonstrate influx-efflux differences quite similar in pattern to those for glucose. The mean F_s and V_m values for efflux were 9.7 and 3.3 times the corresponding values for influx; the mean K_t for influx was three times that for efflux.

Other variables. Two additional comparisons of D-glucose influx and efflux (no trans glucose) were performed in order to control for the technique of washing the cells more rigidly. For influx, the method was as described above (Methods) and involved five washes. For efflux,

the cells were washed five times in precisely the same manner prior to loading with [^{14}C]glucose. After loading, the cells were centrifuged and the packed cell layer tested as described previously.⁴ The results are similar to those in Table II, with mean values for F_s (ml/min per ml cell water), K_s (mM), and V_m ($\mu\text{mol}/\text{min}$ per ml cell water) of 6.8, 3.7, and 25.2, respectively, for efflux, and 0.7, 17.0, and 10.1, respectively, for influx.

Similar results were also obtained with a sample of fresh human blood in place of the 3–6 wk old blood bank specimens. The mean values of F_s , K_s , and V_m for glucose influx (two determinations) were 1.4, 12.0, and 16.7, respectively, as compared with 15.4, 2.6, and 40.0, respectively, for efflux (one determination).

Asymmetric effects of inhibitors. Using an optical method to follow the transfer of glucose, Wilbrandt (20) reported that polyphloreitin phosphate, a highly polymerized impermeable inhibitor, was more effective against efflux than influx. Bowyer and Widdas (21) subsequently developed a mathematical treatment based on the symmetrical mobile-carrier model that accounts for the asymmetric effects on the fluxes of competitive inhibitors which, like polyphloreitin phosphate, are present at different concentrations on the opposite sides of the cell membrane. However, the hypothesis failed to explain their observation that dinitrofluorobenzene, an inhibitor which reacts irreversibly with $-\text{SH}$, $-\text{NH}_2$, and phenolic hydroxyl groups, was also more effective against efflux than influx (21). To explore the question further in the light of the present results, we studied the effects of PMBS, DCDS, and NEM on influx vs. efflux tested in the absence of trans glucose. Fig. 4 illustrates the results with PMBS, a noncompetitive inhibitor which binds $-\text{SH}$ groups reversibly. This mercurial is considered to be relatively impermeant and to react mainly with external $-\text{SH}$ groups (22). Efflux was readily inhibited by 0.025–0.25 mM PMBS and 50% inhibition was observed at approximately 0.18 mM. Influx, in contrast, was inhibited less than 5% on the average in this range.

DCDS, a derivative of diethylstilbestrol, was shown to be an effective inhibitor of glucose efflux by LeFevre (4), who noted that inhibition observed with the synthetic estrogens does not follow typical competitive kinetics. In two experiments the effects of DCDS at final concentrations of 0.005 and 0.05 mM^{*} on influx and efflux at cis glucose concentrations of 61–68 mM were tested. Efflux was inhibited by 56 and 34% at the lower concentration and by 61 and 100% at the higher.

^{*}This procedure involving identical numbers of washes was also followed in the studies with 3-O-methylglucose described in the preceding section.

^{*}At 0.05 mM some DCDS precipitated from the aqueous test buffer. The precise concentration, therefore, was indeterminate at this level.

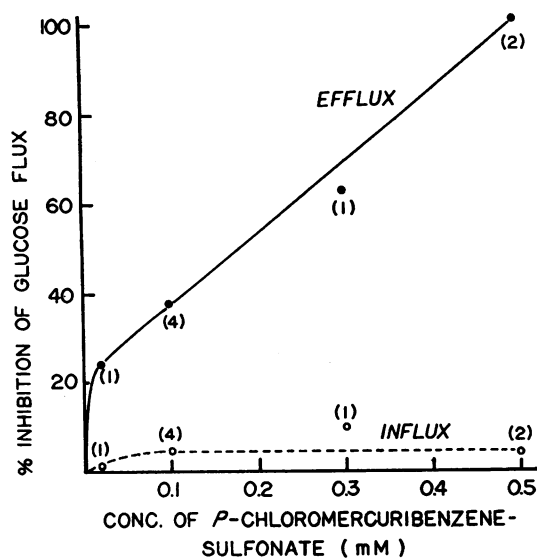


FIGURE 4 Inhibition of glucose efflux (●—●) or influx (○---○) by various concentrations of *p*-chloromercuribenzenesulfonate. Fluxes were estimated without trans glucose as described in the text. Numbers in parentheses are the number of observations with separate blood samples.

In contrast, no inhibition of influx was observed with 0.005 mM DCDS and 21 and 26% inhibition was noted at the higher concentration.

The effects on both fluxes of prior treatment with NEM, a compound which binds $-\text{SH}$ groups covalently, in the presence or absence of glucose are summarized in Table VI. The treatment was varied with respect to glucose because Dawson and Widdas (23) reported more rapid inhibition by NEM in the presence of the hexose. The results in Table VI indicate that, as with the preceding compounds, prior treatment with NEM in the presence of glucose inhibited efflux much more than it did influx, i.e., by 69.5 vs. 24.7% ($P < 0.001$). In addition, the presence of glucose during reaction with NEM influenced the subsequent values for efflux much more than those for influx. The values for NEM inhibition of efflux without and with glucose, respectively, were 36.0 and 69.5% ($P < 0.02$), and the corresponding values for influx were 22.5 and 24.7%.

DISCUSSION

The present results confirm the initial report (5) of influx-efflux asymmetry in the mechanism for facilitated diffusion of hexoses across human erythrocyte membranes. The molecular basis of the asymmetry remains a matter of interpretation, and to develop a useful hypothesis it is instructive first to consider portions of the data and subsequently the results as a whole.

If the present study relied solely on efflux estimations, as has often been the case in prior reports, the observa-

TABLE VI
Inhibition of Efflux or Influx of [^{14}C]glucose following Treatment
with *N*-ethylmaleimide in the Presence
or Absence of Glucose*

Treatment	% Inhibition owing to NEM (mean values)		SE	P
	Efflux	Influx		
NEM minus glucose	36.0 (5)	22.5 (6)	10.6	NS
NEM plus glucose	69.5 (8)	24.7 (9)	8.6	<0.001

* Fluxes were estimated in the absence of trans sugar using cis concentrations of 60–65 mM. Numbers in parentheses give the number of estimations with separate blood samples. Standard error (SE) and *P* values refer to efflux-influx differences. For efflux alone, the difference with and without glucose was significant ($P < 0.02$) with SE = 14.0.

tions could be accommodated by the general mobile-carrier model (Appendix). The F_s constants for efflux with and without trans equilibrium glucose were approximately equal, and the mean ratio of B_s/K_{11} was 4.0 (Table II), consistent with reaction rate constant ratios (Table I) of $k_2/k_4 > 7$ and $k_3/k_4 > 8$. According to this interpretation the mobility of the unloaded carrier (k_4) is much less than that of the loaded carrier (k_2), as previously discussed by others (17, 18). The mean value of 41 mM for B_s (15° C) represents an estimate of the carrier-glucose dissociation constant under the assumptions noted in the Appendix.

If one considers in addition the results of the influx estimations, carefully controlled to permit comparisons with efflux, the observations remain explicable by the model in the Appendix but more assumptions are necessary. Intracellular glucose and 3-*O*-methylglucose increased the observed F_s values approximately 7- and 10-fold, respectively. Inspection of equation 3 in the Appendix, which defines F_s , indicates that intracellular hexose might be assumed to alter either the number of available carriers, or certain of the reaction rate constants of the carrier cycle, or both. Assuming that only the number of available carriers changed, the observed ratio of half-saturation constants for glucose influx/efflux, $K_{10}/K_{11} = 2.6$, would indicate that $k_{-1}/k_3 < 0.4$. That is, the reaction rate constants for either dissociation or formation of the hexose-carrier complex would be approximately 2.5-fold greater at the inner as compared with the outer surface of the human erythrocyte membrane. The possibility of an asymmetric carrier mechanism in this sense was posed by Geck (24) in a recent theoretical paper. Lacko, Wittke, and Kromphardt (25) estimated kinetic constants for glucose influx and con-

cluded that an asymmetric model would more adequately explain their observations.

Taken as a whole the observations described in the body of this report point to an inherent difficulty, previously unrecognized, in kinetic studies of the human erythrocyte mechanism. Intracellular hexose, which is required to estimate efflux under all conditions, or influx in the presence of trans sugar, itself appears to alter profoundly the characteristics of the transfer mechanism. This conclusion rests not only on the observed values for the kinetic parameters, F_s and K_{10}/K_{11} , but on the effects of various inhibitors observed by us and prior authors (21). When tested in the absence of trans glucose, PMBS and NEM, reversible and irreversible -SH binding reagents, respectively, which are noncompetitive inhibitors (4), decrease efflux much more markedly than influx. Similarly, Bowyer and Widdas observed that dinitrofluorobenzene, an irreversible, noncompetitive inhibitor, decreased efflux more than influx (21). In addition, glucose increased markedly the reaction of NEM with the transport system as indicated by subsequent assay of efflux rates, whereas the corresponding influx assays showed relatively little effect of glucose. Finally, 0.005 mM DCDS, a derivative of diethylstilbestrol which is not a sulfhydryl binding reagent, markedly decreased efflux with no effect on influx.

The present kinetic data and the effects of the various inhibitors suggest the hypothesis that the hexose carrier system in human erythrocyte membranes exists in two states. *Carrier state I* corresponds to the condition of no intracellular hexose, and in this state the transport parameters include a relatively high K_{11} , low F_s , and low V_m ; the transfer system is relatively insensitive to inhibitors such as PMBS, NEM, DCDS, and dinitrofluorobenzene; and glucose has little effect on the reactivity toward NEM. *Carrier state II* results from the presence of intracellular hexose, and the transport parameters include a relatively low K_{11} , high F_s , and high V_m ; the transfer system is relatively reactive to the inhibitors above; and glucose markedly increases the reactivity toward NEM.

The "two-carrier state" hypothesis, which appears the most plausible in view of all the present evidence, implies that the single carrier model (Appendix) cannot be used to interpret observations comparing hexose-loaded with hexose-depleted cells. It does not exclude the possibility, however, that under a defined condition the cells could have all the membrane carriers in the same state, and these could function via a cycle of reactions such as postulated in the Appendix. The individual reaction rate constants need not be identical, i.e., in a given carrier state the facilitated diffusion could occur via an asymmetric cyclic mechanism.

The two carrier states I and II could represent two separate transport systems in the membrane. Inasmuch as a wide variety of inhibitors, including the compounds tested above and others such as phloretin and diethylstilbestrol, will affect influx (no trans hexose) as well as efflux if sufficiently high concentrations are used, it seems plausible to assume one membrane system which may exist in different states. Conversion from state I to II could involve a change in the total concentration of functional units as well as in the chemical reactivity of each unit. Inasmuch as hexose transport is dependent on certain external sulfhydryl groups (22), it is likely that the transport involves membrane proteins and that functional transitions could result from conformational changes and perhaps allosteric effects (9, 26). The observation that intracellular 3-*O*-methylglucose, a non-metabolized hexose, influences the kinetic parameters in a manner similar to internal D-glucose indicates that hexose metabolism is not required for the proposed biochemical transition in the transfer system.

A complete kinetic analysis of the type described above was performed by Regen and Morgan (6) using rabbit erythrocytes and 3-*O*-methylglucose at 37°C. They interpreted their results to indicate that there is no significant influx-efflux asymmetry and no effect of trans hexose on the flux rates in this species. Recent studies in our laboratory support these conclusions.⁶ The rabbit mechanism thus appears to be a symmetrical facilitated diffusion. Comparison of their observed maximal transfer rates at 37°C with the present observations at 15°C (Table V) demonstrates that the human mechanism is much more rapid. For example, efflux and influx with no trans 3-methylglucose are, respectively, at least 375 and 16 times greater for the human as compared with the rabbit erythrocytes. On the other hand, the corresponding half-saturation constants for efflux and influx, respectively, show less species difference with observed values of 4.8 and 3.5 mM for the rabbit and 2.0 and 5.6 for the human.

The erythrocyte has been a favorite cell for studying hexose transport, owing, in part at least, to the presumed simplicity of the process, a simplicity which may hold for the rabbit but not for the human cell. Indeed, in recent years a number of authors have found the symmetrical mobile-carrier model of Widdas (1) inadequate and several new models have been proposed (8, 9). The evidence presented in this report suggests that models developed on the basis of the kinetic evidence available heretofore may be severely limited, because the procedures for estimating fluxes change the transport mechanism. Moreover, marked species differences exist and should be taken into account. A more fruitful approach

⁶ Batt, E. R., J. Raskin, and D. Schachter. Unpublished observations.

to the molecular basis of such membrane processes appears to be the increasing application of biochemical methodology in combination with kinetic studies of a variety of cell types.

APPENDIX

The mobile-carrier model as formulated by Regen and Morgan (6) is illustrated in Fig. 5. Assuming a steady state of the membrane constituents during any period of flux estimation, they derive the following general equations for efflux (V_e) and influx (V_i):

$$V_e = \frac{S_i F_s}{1 + S_i/B_s + \frac{(S_i - S_o) M_{si}}{1 + S_o/R_s}} \quad (1)$$

$$V_i = \frac{S_o F_e}{1 + S_o/B_s + \frac{(S_o - S_i) M_{so}}{1 + S_i/R_s}} \quad (2)$$

The symbols used are defined as follows: S_i and S_o are intracellular and extracellular sugar concentrations, respectively;

$$F_s = C_t \cdot \frac{k_{-1}k_{-2}k_{-3}k_{-4}}{(k_2k_3 + k_{-1}k_{-2} + k_{-1}k_3)(k_4 + k_{-4})} \quad (3)$$

C_t = total carrier concentration at the interfaces;

$$B_s = \frac{k_{-1}k_{-2}(k_4 + k_{-4})}{k_1k_4(k_2 + k_{-2})} = \frac{k_3k_2(k_4 + k_{-4})}{k_{-3}k_{-4}(k_2 + k_{-2})} \quad (4)$$

$$M_{si} = 1/K_{ti} - 1/B_s$$

$$K_{ti} = \frac{(k_4 + k_{-4})(k_2k_3 + k_3k_{-1} + k_{-1}k_{-2})}{k_{-3}(k_{-2}k_{-1} + k_{-2}k_{-4} + k_{-1}k_{-4} + k_{-4}k_2)} \quad (5)$$

$$M_{so} = 1/K_{to} - 1/B_s$$

$$K_{to} = \frac{(k_4 + k_{-4})(k_2k_3 + k_3k_{-1} + k_{-1}k_{-2})}{k_1(k_2k_3 + k_2k_4 + k_3k_4 + k_4k_{-2})} \quad (6)$$

$$R_s = 1/(1/K_{ti} + M_{so}) = 1/(1/K_{to} + M_{si}).$$

In the absence of trans sugar equations 1 and 2 reduce to:

$$\frac{V_e}{(\text{no trans})} = \frac{S_i F_s}{1 + S_i/K_{ti}} \quad (7)$$

$$\frac{V_i}{(\text{no trans})} = \frac{S_o F_s}{1 + S_o/K_{to}} \quad (8)$$

In the presence of trans equilibrium sugar equations 1 and 2 reduce to:

$$\frac{V_e}{(\text{trans})} = \frac{S_i F_s}{1 + S_i/B_s} \quad (9)$$

$$\frac{V_i}{(\text{trans})} = \frac{S_o F_s}{1 + S_o/B_s} \quad (10)$$

Equations 7-10 are similar in form to the Michaelis-Menten

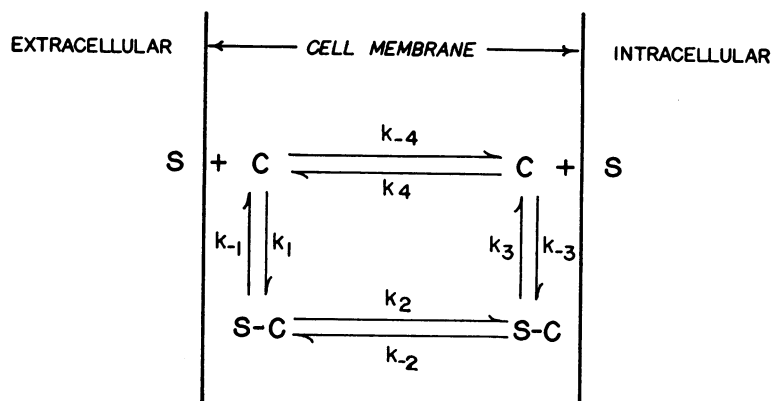


FIGURE 5 Mobile-carrier model showing the reaction rate constants as defined by Regen and Morgan (6). S, C, and S-C represent a sugar molecule, membrane carrier, and sugar-carrier complex, respectively. For influx the reaction rate constants are positive, i.e., k_1 , k_2 , k_3 , and k_4 , and they are correspondingly negative for efflux. For an equilibrating mechanism the overall $K = (k_1 k_2 k_3 k_4) / (k_{-1} k_{-2} k_{-3} k_{-4}) = 1$. The dissociation constant of the carrier sugar complex is k_{-1}/k_1 at the outer surface and k_3/k_{-3} at the inner surface.

equation, with K_i and B_s representing the half-saturation constants. The maximal velocity (V_m) is given by the following: for efflux, no trans sugar,

$$V_m = F_s K_{ti} \quad (11)$$

for influx, no trans sugar,

$$V_m = F_s K_{to} \quad (12)$$

for either flux into equilibrium trans sugar,

$$V_m = F_s B_s \quad (13)$$

(trans)

The constants K_i , F_s , B_s , and V_m are evaluated by plots of S/V vs. S (6) or $1/V$ vs. $1/S$ (14).

The Regen and Morgan treatment above can be extended to deduce the relationships k_{-1}/k_3 , k_2/k_4 , k_3/k_4 , and k_{-1}/k_4 . To simplify the derivations we adopt the plausible assumption that $k_2 = k_{-2}$, and $k_4 = k_{-4}$. These rate constants are assumed to govern readily reversible translocations of loaded and unloaded carriers, respectively, in the membrane. From the preceding assumption and the necessary condition for an equilibrating mechanism, i.e., $k_1 k_2 k_3 k_4 = k_{-1} k_{-2} k_{-3} k_{-4}$, it follows that $k_1/k_{-3} = k_{-1}/k_3$.

Relationship of $k_{-1}/k_3 (= k_1/k_{-3})$. This is deduced from the ratio K_{to}/K_{ti} .

From equations 5 and 6,

$$\frac{K_{to}}{K_{ti}} = \frac{k_{-3}(k_{-2}k_{-1} + k_{-2}k_{-4} + k_{-1}k_{-4} + k_{-4}k_2)}{k_1(k_2k_3 + k_2k_4 + k_3k_4 + k_4k_{-2})}$$

If $k_2 = k_{-2}$ and $k_4 = k_{-4}$, and since $k_{-3}/k_1 = k_3/k_{-1}$,

$$\frac{K_{to}}{K_{ti}} = \frac{k_3(k_2k_{-1} + k_2k_4 + k_{-1}k_4 + k_4k_2)}{k_{-1}(k_2k_3 + k_2k_4 + k_3k_4 + k_4k_2)}$$

Rearranging, dividing all terms by $2k_2k_3k_4$, and transposing,

$$\frac{k_{-1}}{k_3} = \frac{1}{K_{to}/K_{ti}} + \left[\frac{1}{K_{to}/K_{ti}} - 1 \right] \frac{k_{-1}(k_2 + k_4)}{2k_2k_4} \quad (14)$$

From equation 14, if $K_{to}/K_{ti} = 1$, $k_{-1}/k_3 = 1$; if $K_{to}/K_{ti} > 1$, $k_{-1}/k_3 < 1/(K_{to}/K_{ti})$; if $K_{to}/K_{ti} < 1$, $k_{-1}/k_3 > 1/(K_{to}/K_{ti})$.

Relationship of k_2/k_4 and k_3/k_4 . These are deduced from the ratio B_s/K_{ti} . From equations 4 and 5 and using the assumption above,

$$\frac{B_s}{K_{ti}} = \frac{k_3(k_2k_{-1} + k_2k_4 + k_{-1}k_4 + k_4k_2)}{2k_4(k_2k_3 + k_3k_{-1} + k_{-1}k_2)} \quad (15)$$

Rearranging, dividing all terms by $k_{-1}k_3k_4$, and transposing

$$\frac{k_2}{k_4} = \left[\frac{2B_s}{K_{ti}} - 1 \right] + \frac{2k_2}{k_{-1}} \left[\frac{B_s}{K_{ti}} - 1 \right] + \frac{k_2}{k_3} \left[\frac{2B_s}{K_{ti}} \right] \quad (16)$$

From equation 16, if $B_s/K_{ti} \geq 1$, $k_2/k_4 > (2B_s/K_{ti} - 1)$. To solve for k_3/k_4 equation 15 is manipulated similarly except that all terms are divided by $k_{-1}k_2k_4$ and the final result is

$$\frac{k_3}{k_4} = \frac{2B_s}{K_{ti}} + \frac{2k_3}{k_{-1}} \left[\frac{B_s}{K_{ti}} - 1 \right] + \frac{k_3}{k_2} \left[\frac{2B_s}{K_{ti}} - 1 \right] \quad (17)$$

from which it follows that for $B_s/K_{ti} \geq 1$, $k_3/k_4 > 2B_s/K_{ti}$.

Relationship of k_{-1}/k_4 . This is deduced from B_s/K_{to} by analogous steps to those described above, and the result is

$$\frac{k_{-1}}{k_4} = \frac{2B_s}{K_{to}} + \frac{2k_{-1}}{k_3} \left[\frac{B_s}{K_{to}} - 1 \right] + \frac{k_{-1}}{k_2} \left[\frac{2B_s}{K_{to}} - 1 \right] \quad (18)$$

from which it follows that for $B_s/K_{to} \geq 1$, $k_{-1}/k_4 > 2B_s/K_{to}$.

Significance of B_s . From equation 4 and the assumption that $k_2 = k_{-2}$ and $k_4 = k_{-4}$, $B_s = k_{-1}/k_1 = k_3/k_{-3}$. That is, B_s , the half-saturation constant for flux into trans equilibrium sugar, is the dissociation constant of the carrier-sugar complex.

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