Tracer Exchange *vs.* Net Uptake of Glucose through Human Red Cell Surface

New evidence for carrier-mediated diffusion

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ABSTRACT Previous kinetic studies of net sugar movements through the human erythrocyte surface (in response to concentration gradients) have led to postulation of a special "carrier" system for transfer of monosaccharides in these cells. But alternatively some sort of non-specific depression of cell permeability at high sugar concentrations has been suggested as a possible basis for the saturation kinetics and the competitive phenomena observed. New theoretical calculations show that these two interpretations predict entirely different orders of magnitude for the relative rate of tracer glucose exchange at such high sugar levels. Therefore, the speeds of gross chemical equilibration and of tracer glucose equilibration were compared by means of serial analyses on quickly separated cells and media, in thick red cell suspensions. Glucose was first added to glucose-free suspensions, and its entry into the cells followed; then C^{14}-glucose was added after attainment of chemical equilibrium, and the tracer equilibration similarly followed. The speed of the tracer movement in relation to the speed of net uptake was on the order of 50 to 100 times greater than would be found in an uncomplicated diffusion process, regardless of what depressant effect might be occasioned by the high sugar levels. In contrast, the comparative rates observed are predicted by the previously proposed facilitated-diffusion mobile-carrier model for monosaccharide transfer, if the glucose-carrier complex is assigned a dissociation constant (at 20°C.) in the neighborhood of 1 mM.

INTRODUCTION

In recent years, increasing attention has been directed, in a variety of mammalian tissues, to special cell surface properties which appear to mediate the passage of simple sugars back and forth between the cell interior and the interstitial fluid, plasma, or artificial bathing medium. The first evidence that cellular penetration might involve a reversible bonding or complex formation

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between the sugars and a limited number of sites on the cell surface was developed in studies on human erythrocytes (4, 5); and this same system, particularly in its handling of the blood sugar, D-glucose (8, 22–24), has provided the prime example of what is now generally termed "facilitated diffusion" (3). The observation of a transient "uphill" transport by this mechanism in response to counterflow of a competing sugar (17) has demonstrated that the responsible membrane component is mobile (free to diffuse) within the substance of the membrane, and thus may be legitimately called a "carrier."

A systematic exposition of the relevant observed peculiarities of the glucose movements in these cells is given in the review by Bowyer (1). In all these studies, the process observed (by optical densitometric or chemical analytic means) was the *net* gain or loss of sugar by the cells, in response to a correspondingly directed concentration gradient; the principal argument for postulating a carrier system develops from the kinetics shown by this net movement: its dependence on the intracellular and extracellular concentrations of the transported species and of any competing species. A frequent question arising in unpublished commentary on this work has been the possibility of duplicating such anomalous kinetics by way of a non-specific depression of the cell membrane's permeability properties incurred because of the relatively high sugar concentrations in the experimental media, without involving any mechanism of transport other than classical diffusion through a semipermeable barrier. But the present report will show that this interpretation of the net movements predicts, at such sugar levels, unidirectional glucose fluxes or tracer glucose equilibration rates of a totally different order of magnitude from those expected in the operation of the hypothetical facilitated diffusion carrier system. This question is then resolved by quantitative rate studies on the equilibration of C^{14}-glucose through the human red cell surface, in the absence of net transfer. The data show that any interpretation involving simply alteration of classical "permeability constants" is quite untenable, while the model carrier system developed from the earlier kinetic studies is consistent with the new observations. Independent support is also given to the conclusion that the carrier entity moves about within the membrane.

THEORETICAL BASIS

The contrasting potential kinetics of net transfer by a facilitated diffusion process and by unencumbered diffusion have been formally developed elsewhere, notably by Rosenberg and Wilbrandt (16). If the passage of glucose or other non-electrolyte through the cell surface involves only diffusion (through either aqueous pores or the substance of a homogeneous barrier, or both), the unidirectional fluxes of the solute should be proportional to its

respective activities (essentially, to its concentrations) in the two aqueous phases, inside and outside the cell, except for minor corrections associated with any "solvent drag" or other concurrent migrations. The net movement should therefore be approximately described by the relation:

$$\frac{dS}{dt} = k_D(C_s - S/V), \tag{1a}$$

where S is the quantity of intracellular glucose per unit original cell water volume (conventionally in isosmotic units: here 1 *iso*smolal is taken as equal to 300 milliosmolal), C_s is the extracellular glucose concentration in such isosmotic units, and V is the intracellular water volume expressed as a (dimensionless) fraction or multiple of the original cell water volume. k_D is thus a conductance-like permeability constant compounded of the diffusivity of glucose within whatever passages it follows through the barrier, and the length and total effective area of these passageways. (As defined, k_D has dimensions of inverse time, and is an *intrinsic* constant of the cell species, not referring to any particular quantity of cells.) The factor in parentheses in equation (1*a*) is the difference between external and internal sugar levels; its two terms represent the unidirectional fluxes (influx, $\rho_i = k_D C_s$; efflux, $\rho_e = k_D S/V$).

In contrast, this identity of flux ratio with concentration ratio is not generally predicted by the carrier model which has emerged from study of the net transfer. The simplest model consonant with the observations to date[1] is shown in Fig. 1. A transfer operating as stipulated in the figure would obey the following relation based on the diffusion of the carrier-sugar complex within the surface layer:

$$\frac{dS}{dt} = k_C\left[\frac{C_s}{C_s + K} - \frac{S/V}{S/V + K}\right]. \tag{1b}$$

Here k_C is also an intrinsic constant of the cells (dimensions: concentration/time), and represents a diffusion constant compounded of the diffusivity of the complex in the membrane and the fixed concentration of carrier sites present (thus far no means of separately measuring either of these factors has come to light). The sugar-carrier complex's dissociation constant, K, varies systematically with the sugar's molecular structure (9). As can be seen by inspection

[1] This model is that adopted by Widdas (21), and is essentially identical with Rosenberg and Wilbrandt's Type E (16); the simpler but asymmetric model proposed by LeFevre and LeFevre (8) has since been abandoned (7) by reason of the new evidence of the carrier's mobility in the membrane, and the slight but consistent refinement with the Widdas model in fitting the observed time-course of net glucose exit from glucose-laden cells transferred to relatively glucose-deficient media. Moreover, the failure at very high levels which was pointed out in the LeFevre and LeFevre model is *not* apparent in application of equation (1*b*).

of equation (1*b*), it is the order of magnitude of this K in relation to the sugar concentrations (C_s and S/V) which determines the nature of the concentration-dependence of the net sugar transfer. If K is considerably larger than C_s or S/V, equation (1*b*) assumes the form of equation (1*a*), such that $k_C = k_D K$. But for D-glucose at body temperature, K appears to be on the order of 6 to 8 mM (6, 23), considerably less than the levels ordinarily used in the experimental media. Under these circumstances, once the cells have acquired sugar to a level appreciably exceeding K, the efflux term on the right of equation (1*b*) becomes nearly equal to the influx term; thus the resultant net transfer is but a small fraction of the unidirectional fluxes.

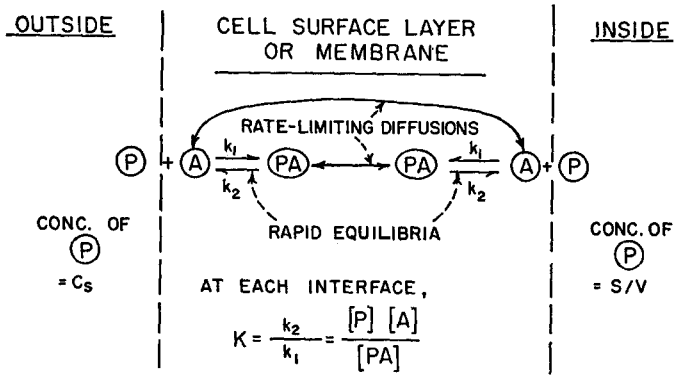

FIGURE 1. Model of facilitated diffusion system. The penetrant P as such is unable to enter the cell membrane, but it combines reversibly with carrier A at either interface, rapidly approaching equilibrium defined by dissociation constant, K. The slower diffusion of complex PA through the membrane, according to its concentration gradient within the membrane, determines the over-all migration of P, as specified in equation (1*b*). No work is done; no metabolic sustenance need be involved.

In application of these considerations to the experiments to be reported here, it is convenient to convert the equations to new terms reflecting the actual parameters determined. These experiments involve equilibration upon addition of glucose to cells suspended at substantial hematocrit readings in an isotonic balanced salt solution, so that C_s, S, and V all vary concomitantly. Moreover, there are three alterable quantities which in any one experiment are fixed: the amount of cells, the amount of medium, and the amount of glucose used. For the analysis, these may be represented in the following three independent constants:

I = fraction of total water which is intracellular at isotonicity;
W = total volume of water in the entire mixture (in any convenient unit);
and Q = total glucose in the entire mixture, in units which would constitute an isosmotic solution in the unit volume of water.

(Q can be treated as constant, because the rate at which glucose is metabolized by the cells in such experiments is quantitatively insignificant.)

The quantities varying with time are then:

S = that part of Q which is intracellular;
V = that part of W which is intracellular;
and C_s, as originally defined = $(Q - S)/(W - V)$, and will now be eliminated by this substitution.

Since now the sugar contents, S and Q, are expressed as *quantities* in the *mixture* rather than as *concentrations* in the isotonic *cell water* volumes, the corresponding conversion factor, WI, must be inserted in equations (1*a*) and (1*b*) in order that the rate constants (k_D and k_C) may retain their original intrinsic significance (remaining independent of fortuitous differences in W and I in different experiments). Accordingly, in the new symbols, equations (1) may be restated:

$$\frac{dS}{dt} = k_D WI\left[\frac{Q - S}{W - V} - \frac{S}{V}\right], \tag{2a}$$

and

$$\frac{dS}{dt} = k_C WI\left[\frac{\dfrac{Q - S}{W - V}}{\dfrac{Q - S}{W - V} + K} - \frac{\dfrac{S}{V}}{\dfrac{S}{V} + K}\right]. \tag{2b}$$

Either of the remaining variables may now be eliminated, because of certain simplifying considerations. The equilibration of water through the cell surface is so much more rapid than the sugar movements that for the purposes of this analysis it is, as usual, treated as instantaneous. Moreover, human red cell water volumes do not deviate conspicuously from perfect osmometric behavior in such mixtures (10, and unpublished studies in this laboratory), so that a given mixture's osmotic pressure is essentially invariant with time, and homogeneous throughout the extracellular and intracellular aqueous phases. Therefore, the cell water volume at any moment is given by the relation, $V = (WIC_m + S)/C$, where C = the total isosmolality of the mixture, and C_m = that of the plain sugar-free, isotonic medium = unity concentration. Thus $Q = W(C - C_m)$; and $S_e = WI(C - C_m)$ = the value of S at equilibrium ($t = \infty$). It will also simplify later expressions to employ the following abbreviations:

$C_i = IC_m$ = tonicity of original cell solutes if dissolved in *total* mixture water;

$$\alpha = C + K;$$

and

$$\beta = C(\alpha - C_m).$$

(Thus C_i is *numerically* equal to I, but is a concentration; it is the tonicity which the original cell solutes would produce if dissolved in the *total* mixture water. α and β have dimensions of concentration and concentration^2 respectively, but they have no physical connotation.) With these substitutions in equations (2*a*) and (2*b*), the transfer rates may be expressed as functions of the single variable, S:

$$\frac{dS}{dt} = k_D W^2 C_i C \left[\frac{S_e - S}{-S^2 + (C - 2C_i)WS + (C - C_i)W^2 C_i} \right], \qquad (3a)$$

and

$$\frac{dS}{dt} = k_C K W^2 C_i C \left[\frac{S_e - S}{-\alpha^2 S^2 + (\beta - 2C_i K)\alpha WS + (\beta - C_i K)W^2 C_i K} \right]. \qquad (3b)$$

Upon integration ($t = 0$ when $S = 0$ at the instant of addition of Q), equations (3) yield:

$$k_D C_i C t = \frac{S^2}{2W^2} - \frac{C(1 - I) - C_i}{W} S - (1 - I)IC^2 \ln (1 - S/S_e), \qquad (4a)$$

and

$$k_C K C_i C t = \frac{\alpha^2 S^2}{2W^2} - \frac{\beta(1 - I) - C_i K}{W} \alpha S - (1 - I)I\beta^2 \ln (1 - S/S_e). \qquad (4b)$$

The contrast in these alternative theoretical relations is illustrated by a typical example in Fig. 2; in this instance, the hematocrit reading of the cells in the same volume of plain medium is taken as 50 per cent; and the cell "dead space" is taken at a typical figure, 30 per cent of the hematocrit reading, or 15 per cent of the total volume; thus $WI = 0.35$ times the total mixture volume. The amount of glucose is such that its equilibrium level is isosmotic; *i.e.*, $C = 2$, and numerically $Q = W$, and $S_e = WI$. Since the fraction of water in an isosmotic glucose solution in the regular isotonic salt mixture is about 0.929, $W = 0.790$ times the total volume, so that $I = 0.443$. These numerical substitutions in equations (4) give:

$$k_D t = 0.904S^2 - 0.959S - 1.114 \ln (1 - S/0.35), \qquad (4a')$$

and

$$k_C K t = 0.904(K + 2)^2 S^2 - 0.959(K + 2)(K + 1.66)S - 1.114(K + 1)^2 \ln(1 - S/0.35). \quad (4b')$$

In Fig. 2, K is taken at the usual figure of 0.025 isotone (*i.e.*, 7.5 mM). The heavy curves in the two panels of the figure illustrate the relatively minor

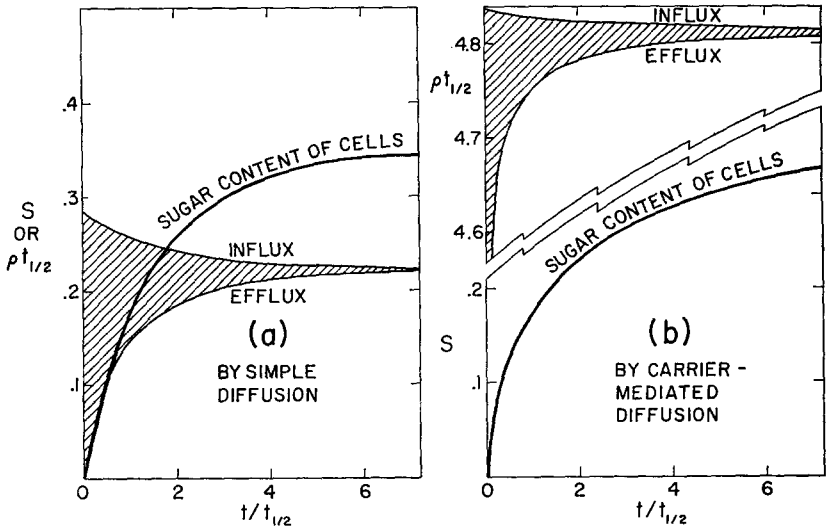

FIGURE 2. Alternative theoretical patterns for glucose fluxes, in typical experimental situation. Here, $H = 0.50$; $D = 0.15$; equilibrium $C_s = 1$; $K = 0.025$. Vertical scale shows both cell sugar content (S) in each unit volume of mixture, and the corresponding fluxes (ρ), with the half-equilibration time as the time unit. Horizontal scale shows time since addition of sugar, in such half-time units. Sugar content curves are as defined by equations ($4a'$) and ($4b'$) in respective panels; fluxes and half-times, as given by corresponding relations in Table I. Note large break in scale in panel (b), necessitated by the much higher relative magnitude of the fluxes.

differences between the predictions of relations ($4a'$) and ($4b'$) in regard to the time-course of the sugar uptake. Although, as will be detailed later, it is possible to show that the observed uptake patterns accord with type (b) rather than with type (a), this quantitative distinction is not overwhelming, as Fig. 2 illustrates. On the other hand, the two hypotheses predict altogether different orders of relative magnitude for the unidirectional fluxes which result in this net uptake. This is shown in Fig. 2 by the respective flux curves (the enclosed cross-hatched areas thus also give the net accumulation of S as plotted by the heavy curves). Paralleling this very large difference between the alternative theoretical predictions as to the magnitude of the fluxes, the carrier interpretation correspondingly predicts a vastly more rapid equilibration of tracer

glucose, added after attainment of chemical glucose equilibrium, than is given by the diffusion interpretation (in the example of Fig. 2, more than 21 times as fast, in terms of the half-time of the net uptake). The respective relations are summarized in Table I.

TABLE I

ALTERNATIVE THEORETICAL RELATIONS DEFINING PRINCIPAL PARAMETERS

Parameters	By simple diffusion	
	General expressions	Values in example of Fig. 2
Influx, ρ_i	$k_D WC \dfrac{W(IC-C_i)-IS}{W(C-C_i)-S}$	$0.700k_D \dfrac{0.790-S}{1.23-S}$
Efflux, ρ_e	$k_D WC \dfrac{IS}{WC_i+S}$	$0.700k_D \dfrac{S}{0.350+S}$
Net uptake half-time	$\dfrac{0.125(IC-C_i)(IC+3C_i)+C(1-I)(0.193IC+0.5C_i)}{C_iCk_D}$	$\dfrac{0.632}{k_D}$
Fraction of tracer in cells at time t	$I(1-e^{-k_D t/(1-I)})$	$0.443(1-e^{-k_D t/0.557})$
Tracer equilibration half-time	$\dfrac{0.693(1-I)}{k_D}$	$\dfrac{0.386}{k_D}$
Relative half-times (tracer/net)		**0.610**

Parameters	By carrier-mediated diffusion*	
	General expressions	Values in example of Fig. 2
Influx, ρ_i	$k_C WC \dfrac{W(IC-C_i)-IS}{W(\beta-C_iK)-\alpha S}$	$0.700k_C \dfrac{0.790-S}{1.61-2.025S}$
Efflux, ρ_e	$k_C WC \dfrac{IS}{WC_iK+\alpha S}$	$0.700k_C \dfrac{S}{0.00875+2.025S}$
Net uptake half-time	$\dfrac{0.125(I\beta-C_iK)(I\beta+3C_iK)+\beta(1-I)(0.193I\beta+0.5C_iK)}{C_iCKk_C}$	$\dfrac{14.09}{k_C}$
Fraction of tracer in cells at time t	$I(1-e^{-k_C t/(1-I)(C_s+K)})$	$0.443(1-e^{-k_C t/0.571})$
Tracer equilibration half-time	$\dfrac{0.693(1-I)(C_s+K)}{k_C}$	$\dfrac{0.396}{k_C}$
Relative half-times (tracer/net)		**0.0281**

* Transformation from system (a) to system (b) in this table, as well as in equations (3) and (4), is effected by the following substitutions in the denominators of rate expressions, or in the numerators of time expressions: for C, substitute β; for C_i, substitute C_iK; for S, substitute αS; for t, substitute Kt.

TECHNICAL

Materials Human blood obtained by venipuncture was occasionally defibrinated with glass rods, but more generally collected into standard EDTA platelet packs (Fenwal), and stored at 2–4°C. when not used the same day. The donors were frequently polycythemic patients undergoing therapeutic phlebotomy, but no bearing of this disease state on the experimental system was noted. Just prior to use, cells separated by centrifugation were taken through at least three successive washes in relatively large volumes of normal saline (Baxter), and finally two or three washes in the experimental medium before final suspension therein. In the course of this procedure, the major part of the white cells was discarded with the uppermost red cell layers.

The medium routinely used was prepared by mixing the following relative volumes of aqueous solutions, each adjusted to isosmolality (taken as 300 milliosmoles/kg. of water, as determined with the Fiske osmometer): 100 parts NaCl, 4 parts KCl, 3 parts $CaCl_2$, and 2 parts $MgCl_2$; of this mixture, 4 parts were then added to one part tris(hydroxymethyl)aminomethane (tris), adjusted with HCl to pH 7.4. This procedure gave the following approximate concentrations in the final medium: tris, 35 mM; Na^+, 119 mM; K^+, 4.8 mM; Ca^{++}, 2.6 mM; Mg^{++}, 1.7 mM; pH 7.4; isotonic within 0.5 per cent. Glucose stock solutions were prepared by dissolving anhydrous D-glucose (Fisher) or uniformly labeled C^{14}-glucose (Isotopes Specialties) directly in this medium, so that in all mixtures the non-glucose components were at unit tonicity.

Basic Design of Experiments Since very high speeds of tracer equilibration were anticipated and since the temperature coefficient of the process is known to be fairly high (5), the incubation temperature for the experiments was set down to about 20°C. instead of the 37°C. used in nearly all the previous work with net movements. Usually, 10 to 30 ml. of a cell suspension at 50 to 70 per cent hematocrit reading was mixed, at "zero" time, with a substantially smaller volume of a glucose stock solution (usually containing 100 mg./ml.). Homogeneity was maintained by frequent manual mixing, and serial samples withdrawn at appropriate intervals. The medium and cells of these samples were at once separated as detailed below, for subsequent glucose analysis (in a few earlier experiments, only the supernatant preparations were analyzed). A duplicate of the experimental mixture was meanwhile kept at 37°C. to assure virtually complete equilibration of the sugar throughout the intra- and extracellular water by the time the above phase of the experiment was terminated. After determination of the equilibrium sugar level as explained below, a stock of glucose including the tracer was prepared at the same concentration, and a small proportion of this added to portions of the second (equilibrated) mixture after it was brought down to 20°C. Samples taken at intervals from such mixtures were then handled in the same manner as in the preceding chemical equilibration phase of the experiment, but in this case the primary analyses were those concerned with the C^{14} distribution, since there was no appreciable net movement of glucose. Comparison of the two phases of the experiment thus gave the desired information on the comparative rates of gross chemical equilibration and tracer equilibration.

Estimation of Fixed Parameters Determination (with the Fiske osmometer) of the osmotic pressure of the experimental mixtures, and of corresponding controls lacking the sugar, or lacking the cells, permitted the estimation of the water content of the mixtures, by means of the following relations:

Let Π_X and π_X designate respectively the total osmolality of any mixture X, and the *iso*smolality of its glucose component (thus $\pi_X = Q_X/W_X$ = the equilibrium C_S). Let subscripts have the following significance: S, the stock glucose solution; G, the cell-free control mixture; E, the experimental mixture; and M, the medium. The measured quantities are then Π_S, Π_G, Π_E, and Π_M,[2] which are converted to the respective π's by the relation, $\pi_X = \Pi_X/\Pi_M - 1$. Then

$$W_E = \frac{\pi_G \pi_S W_M}{\pi_E(\pi_S - \pi_G)}; \tag{5}$$

the amount of water per unit volume of the standard medium was determined by direct weighing, vacuum oven-drying, and reweighing, to be 0.989, so that W_M, the amount of water in the medium used in mixing the cell-free control, is 0.989 times the volume of medium used. A check on the volumetry and other experimental manipulations is then given by the relation:

$$Q = \frac{0.989\pi_S\pi_G}{\pi_S - \pi_G} \times \text{the volume of the mixture}; \tag{6}$$

this invariably agreed within 0.5 per cent with the Q calculated from the original weighing of the anhydrous glucose reagent and the volumes taken for the mixtures.

Original total cell volumes as hematocrit readings (H) were determined with the Parpart and Ballentine air-driven high speed microhematocrit (12); equilibrium hematocrit readings in the mixtures were also secured as checks. Cell "dead space" (D) (in hematocrit units) in the original suspension (O) is then given by the relation:

$$D_O = \frac{0.989\pi_S(\pi_E - \pi_G)}{\pi_E(\pi_S - \pi_G)} + 0.011H_O. \tag{7}$$

And, since $I_E = V_O/W_E = (H_O - D_O)W_M/0.989W_E$,

$$I_E = 1 - \frac{\pi_E}{\pi_G} + H_O\,\pi_E\left[\frac{1}{\pi_G} - \frac{1}{\pi_S}\right]. \tag{8}$$

Since $S_{eE} = I_E Q$, and $C_E = C_M + Q/W_E$, all the parameters in equations (4) are known so as to define S as a function of $k_D t$ or of $k_C t$ and K, according to the respective hypotheses.

For the tracer experiments, the corresponding simple mixing relations are used to secure numerical estimates of Q, W, and I in the final mixture, in order to evaluate the apparent k_D and k_C from the experimental rate of equilibration.

Sampling and Cell Separation The high speed of transfer, and especially of the tracer equilibration, made ordinary centrifugal separation of cells from media inadequate for temporal resolution even at the reduced temperature of 20°C. A few

[2] The osmotic pressure of the cell suspension without glucose was also checked for isotonicity.

experiments were carried out with the same high speed air-driven microcentrifuge as was used for the hematocrit determinations, which gave reasonably effective separations in less than 30 seconds when the mixtures were added to the spinning rapid separator head (13). However, most samples handled in this manner showed a disturbing degree of hemolysis; moreover, analysis of the media only, and not of the separated cells as well, was feasible by this method.

Therefore, adaptation was made of a method mentioned in the oral report accompanying a related publication (14), which involves the use of ice cold $HgCl_2$ solutions to interrupt the sugar transfer at a specified instant. This "fixes" the glucose compartments sufficiently to allow a reasonable time for a more gentle separation in an ordinary centrifuge. The "fixing mixture" used for most of the experiments had the following approximate composition: NaCl, 310 mM; $HgCl_2$, 2 mM; and raffinose, 5 mM; 10 ml. of this mixture in a 15 ml. centrifuge tube in an ice bath serves to terminate abruptly the transfer of glucose, when a 0.7 to 1.0 ml. sample is added from an experimental cell suspension at room temperature. The instant of this mixing defines the sampling time; conventional centrifugal separation at 0–2°C. during the subsequent 4 to 5 minutes provides the necessary temporal resolution.[3] Immediately at the end of this cold centrifugation, the major part of the supernatant fluid was withdrawn for later analysis, and the rest discarded by decanting as completely as was possible without visible loss of any of the packed cells. Subsequently, the contents of these cells were released by total hemolysis, by dilution to a fixed volume with a solution of Na_2CO_3 (1 gm./liter). This preparation served two purposes: (*a*) glucose analyses gave a direct estimate of S as a function of time, as detailed below; and (*b*) colorimetric reading of the hemoglobin band at 545 mμ (after appropriate dilution in the same Na_2CO_3 solution) obviated the necessity for any volumetric precision in taking the original suspension samples, thus greatly facilitating the handling and proper timing of the samples taken within the first minute after mixing the tracer glucose with the cell suspensions.

The reason for the high concentration of salt in the mercurial fixing mixture (close to double isotonic) is that the high glucose content of the cells during most of the experiment would lead to osmotic hemolysis upon dilution in this mixture if the latter were not sufficiently hypertonic. The addition of the non-penetrating trisaccharide, raffinose, provides a means of estimating the volume of trapped supernatant fluid remaining with the packed cells after decantation[4] (2); (any non-interfering agent which would distribute itself evenly in the extracellular volume could be substituted).

Additional samples were handled in a similar manner except that the NaCl was omitted from the diluent, so that the cells were at once hemolyzed, and the glucose and C^{14}-glucose homogeneously distributed throughout the entire water volume. These served for confirmation of recoverability of the total sugar and radioactivity

[3] Tests of the exclusion of C^{14}-glucose from glucose-laden cells mixed with the tracer in such fixing mixtures showed that the residual fluxes during separation, by the procedure described, corresponded to an apparent delay of less than 1 second in termination of flux.

[4] The volume of trapped fluid actually met in the experiments was typically about three-fourths of the original suspension sample volume.

contents of the mixtures, and as a basis of reference for the degree of equilibration in the serial experimental mixtures.

Analyses Somogyi filtrates (18) were prepared from the Na_2CO_3 hemolysates of the packed cells, and from the corresponding supernatant mixtures after treatment of the latter with a similar amount of osmotic lysate from thoroughly washed human erythrocytes. This added hemoglobin served to carry down, in the Somogyi precipitation, the residual Hg^{++} which otherwise introduced a varying error in the glucose analyses. The filtrates were analyzed for glucose in some early experiments by Nelson's method (11) with Somogyi's modified reagent (19); but for nearly all the work the glucose oxidase method (glucostat, Worthington) was used. Raffinose analyses were carried out with the Roe ketose method (15)[5]; a minor correction of the raffinose analyses was required in accordance with the glucose content. A Coleman Universal spectrophotometer was used for all these analyses.

For the radioactivity determinations, in the earlier experiments the glucose in samples of the Somogyi filtrates was converted to glucosotriazole for liquid scintillation counting, following in detail the procedure described by Steele, Bernstein, and Bjerknes (20), except that the counter and its shielding pig were at room temperature instead of in a deep freeze. This method assures the chemical identity (as glucose) of the material providing the C^{14} to be counted. In the present experiments, however, such assurance is of minor consequence, since the quantities of glucose involved are overwhelmingly greater than the amounts metabolizable by the cells in the course of the tracer experiments; therefore, for most of the work the following vastly simpler preparation was adopted. Aliquots of the Somogyi filtrates were taken directly to dryness in 10 ml. Folin-Wu blood sugar tubes by immersion in a water bath at 80–90°C. and application of a gentle air current. The film of dried material was then redissolved in one drop (*ca.* 0.05 ml.) of water, and taken up in 10 ml. of absolute ethanol. For counting, 5 ml. of this ethanolic solution was mixed directly with 20 ml. of xylene containing the phosphor PBD (2-phenyl-5-(4 biphenylyl)1,3,4-oxadiazole) at 3 gm./liter, and handled in counting as with the more elaborate preparation (20).[6]

With this procedure, the glucose and radioactivity analyses of the packed cell hemolysates (after correction for the content of the trapped supernatant fluid) gave a direct measure of the total cellular contents in the original experimental samples. Similarly, the analyses of the supernatants gave directly the *total* extracellular content of glucose and tracer, without involving the usual assumptions as to the volumes of the extracellular compartment in the suspensions.[7] Each of the several analyses in-

[5] Optical density was read at 402 mμ instead of the recommended 520 mμ, because of the less critical setting at the former (wider) peak; the accompanying loss in sensitivity was trivial.

[6] A minor but essential refinement of the published procedure is the elimination of the rubber serum bottle stoppers used in the counting vial caps, since the liquid counting mixture slowly extracts from these stoppers some material which disturbs the scintillation counting. The alternative adopted (recommended by Dr. R. Steele) is puncturing of the polyethylene caps with a needle to permit capping of the vials, and subsequent resealing with a moderately hot small soldering iron. The levels of radioactivity employed in the experimental suspensions were 0.2 to 0.5 μc./ml., corresponding to about 1 to 8 mμc. in the aliquots ultimately counted.

[7] The volume occupied by the cells *in the fixing mixture* was estimated on the assumption of continued obedience of simple osmotic laws, since this volume was too small to be reflected in the supernatant raffinose analyses. This assumption might introduce an uncertainty of perhaps 0.1 ml. in estimation of the total volume of about 10.5 ml. of the supernatant fixing mixture.

volved is nominally subject to an uncertainty of perhaps 2 to 3 per cent; in practice, however, the total recoveries (cells plus medium), calculated as outlined above rarely deviated by more than 2 per cent from those expected on the basis of the hemoglobin analyses taken as the measure of the original sample volumes.

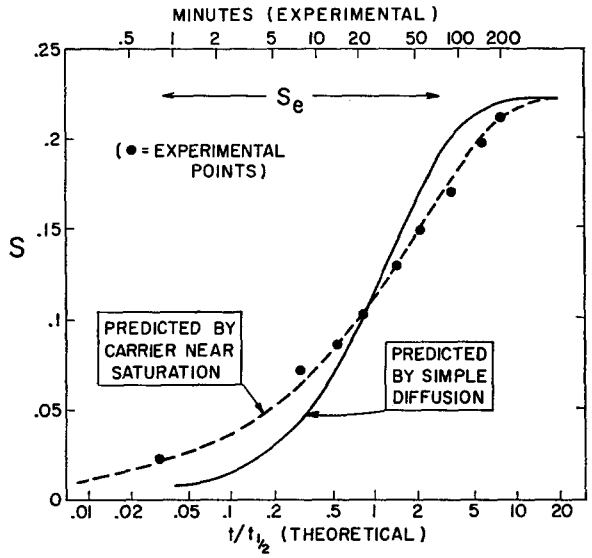

FIGURE 3. Uptake of glucose by human erythrocytes; typical chemical equilibration experiment as described in text. Here, $I = 0.469$; and for each unit volume of mixture, $W = 0.781$, and $Q = 0.475$. Cell sugar content (S), for each unit volume of mixture, is plotted against time on a logarithmic scale. Time scale for experimental points is given at top; that for theoretical relations, in half-time units, at the bottom.

RESULTS

Equilibration of originally glucose-free cells with added glucose was followed in eleven experiments; the equilibrium C_s varied between 0.3 and 0.85 (the initial C_s typically being about 80 per cent greater by reason of the high hematocrit levels used). The cell sugar analyses given in Fig. 3 are illustrative of the uptake patterns found, which were consistent in all experiments; in this instance the equilibrium C_s was 0.608. The fit of the experimental data to the form of equation (4*b*), assuming *any* reasonably small value of K, is illustrated by the good approximation to the *broken* curve. Such semilogarithmic plotting of equations (4) permits shifting the theoretical relations along the time axis in this manner to give the best superimposition on the experimental points, in order to secure numerical estimates of the k's. The value for k_C, however, depends on the figure taken for the affinity constant, K; the relation (from Table I) is in this particular instance:

$$k_C = 0.0178K + 0.0200 + 0.00355/K \text{ (isosmolal units/minute).} \quad (9)$$

In Fig. 3, the *continuous* curve defines the form given by the diffusion equation (4*a*); this gives a poor fit to the data, but if arbitrarily the half-equilibration time of about 25.5 minutes is taken as the basis of reference, a diffusion k_D of 0.0178 per minute can be calculated for comparison with other records.

Similarly, in eight experiments, after such chemical equilibration, tracer glucose was added without appreciable change of C_s, and its equilibration followed. Typical of all the results are those given by the solid symbols in Fig. 4,

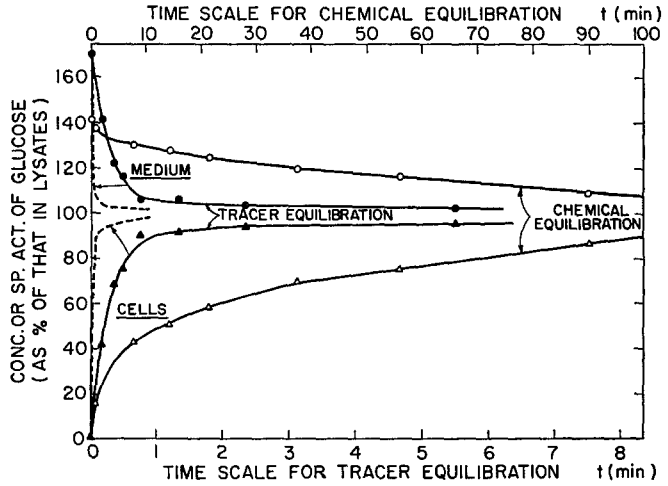

FIGURE 4. Comparative equilibration of tracer glucose and net glucose entry, in same cell suspension. Chemical equilibration experiment is same as that analyzed in Fig. 3; in tracer equilibration phase, $I = 0.411$; $C_s = 0.615$; and for unit volume of mixture, $W = 0.800$. Experimental points, circles denote medium; triangles denote cells; open symbols refer to chemical equilibration experiment (time scale at top); solid symbols refer to tracer equilibration experiment (time scale at bottom). Broken curves are repetitions of tracer equilibration curves on the twelve times reduced time scale of the chemical equilibration, for direct comparison with the latter.

which involves the identical cell suspension characterized in Fig. 3; in fact, the data of Fig. 3 are here re-expressed (open symbols) in a manner facilitating comparison with the tracer equilibration. The tracer movement can be seen to be vastly more rapid than the chemical equilibration which preceded it. (This was equally true in two instances in which equilibration from the opposite direction was studied by dilution of cells, equilibrated with labeled glucose, in a chemically identical, but unlabeled, medium.) Again taking a half-equilibration time estimate as the basis for calculation, one finds k_D in the tracer experiment of Fig. 4 to be on the order of 1.53 per minute, or nearly 100 times that given by the net uptake data; and here again, k_C depends on K, the relation being:

$$k_C = 1.53\ (0.615 + K)\ \text{(isosmolal units/minute)}. \qquad (10)$$

The data can thus be interpreted by the carrier hypothesis, if K is of the magnitude defined by simultaneous solution of relations (9) and (10). Fig. 5 shows the curves defined by these relations, and by corresponding equations given by the only three other experiments in which both the chemical and the tracer equilibrations were performed on identical cell suspensions. (The additional single phase experiments all defined very similar curves.) The designated points of intersection in Fig. 5 thus mark the values for k_C and K at

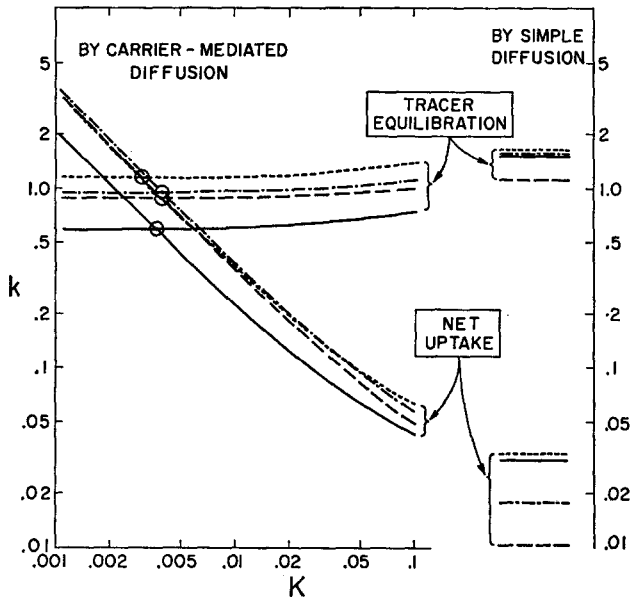

FIGURE 5. Conditions of mutual consistency of chemical and tracer equilibration. Four types of line in each group of records refer respectively to four experiments in which chemical and tracer equilibration were both studied in the same cell suspension. At right, incompatibility of two phases of study as interpreted on basis of simple diffusion is shown by very large discrepancy in k_D's. At left, the four sets of relations like those of equations (9) and (10) are given, in log-log plots of k_C *vs.* K. The designated intersections mark the values at which the observed behavior is consistent with the model of Fig. 1.

which for each case the tracer behavior and net sugar uptake were mutually consistent, as analyzed with the model system adopted in Fig. 1. The order of magnitude implied for k_C is about 1 isosmolal unit per minute, and K is 0.003–0.004 isosmolal units (or about 1 mM). The latter figure is only about one-seventh of that which has been derived from earlier net transfer kinetic analyses. This quantitative discrepancy appears to derive from the lower temperature used in the present study; in preliminary examination of the effect of temperature on the densitometric responses to added glucose, a considerably higher apparent glucose-carrier affinity has been observed at temperatures near 20°C. than at body temperature. This finding modifies the implications

generally attached to the high temperature coefficient of the transfer process, and will be given special consideration in a separate report.

INTERPRETATION

The totally different orders of magnitude of the transfer constant given by the net glucose movements and the tracer glucose equilibration experiments, as analyzed in terms of a diffusion system, render untenable any interpretation of the net transfer kinetics based on a simple diffusion, *whether or not* cell permeability is assumed to vary with sugar concentration. In contrast, the facilitated diffusion model, previously suggested by the saturation kinetics and other aspects of the sugar movements in these cells, *predicts* the observed comparative rates of tracer equilibration and net transfer, given a very high affinity between the glucose and the hypothetical carrier sites. A conceivable alternative explanation could be based on the argument that the high rate of tracer equilibration might derive from a separate *exchange* system which, being incapable of effecting any *net* movement, is not reflected in chemical equilibration experiments. But this would constitute an additional, ill defined, *ad hoc* hypothesis to be appended to that concerning the variable "permeability constant;" whereas the facilitated-diffusion model at once provides a unitary, consistent interpretation of all the peculiarities so far described in the system.

Rosenberg and Wilbrandt (17) have discussed the differences in behavior to be expected in a system of this type and one in which the model membrane provides in essence simply two adsorption layers (the sugar-combining component remaining at fixed positions rather than moving about within the membrane). An adsorption membrane system would display essentially the same flux ratio characteristics as expected in passive diffusion; thus, the present experiments provide independent support to Rosenberg and Wilbrandt's demonstration of the mobility of the carrier entity.

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