Biochimica et Biophysica Acta, 382 (1975) 353–368
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 76895

ZERO-TRANS AND INFINITE-CIS UPTAKE OF GALACTOSE IN HUMAN ERYTHROCYTES

HAGAI GINSBURG and W. D. STEIN

Biophysics Group, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem (Israel)

(Received August 21st, 1974)

SUMMARY

1. The zero-trans and infinite-cis uptake of galactose into human erythrocytes was measured as a function of galactose concentration at 20 °C.

2. A special procedure, the "cis-trans test" has been developed to determine the directionality of an asymmetric transport carrier.

3. Using the "cis-trans test" and results obtained by phloretin inhibition, we could show the existence of two sites mediating galactose uptake. The kinetic parameters of the high affinity site are $K_1 = 11$ mM; $V_1 = 16$ mmol · cell unit^{-1} · min^{-1} and of the low affinity site: $K_2 = 286$ mM; $V_2 = 21$ mmol · cell unit^{-1} · min^{-1}.

4. The infinite-cis K_m, using an integrated rate equation treatment was 21 mM and that found by a direct preloading procedure was 25 mM. The existence of a high affinity site at the inner side of the membrane was thus confirmed.

INTRODUCTION

In a recent extensive review on the present state of the carrier hypothesis, Le Fevre [1] concludes "that the sugar transfer mechanism in human erythrocytes which has served for years as a classical model for carrier mediated transport, now appears to provide the most distinctly dissonant behaviour". Probably part of this dissonance stems from technical difficulties and experimental inaccuracies in the determination of kinetic parameters. But it is also possible, as we have argued previously [2], that the system is not a simple carrier.

Recent suggested mechanisms are of great diversity. Among those which are extensions of the carrier mechanisms one might list Lacko et al. [3], whose data suggested an asymmetrical carrier system with a higher affinity at the outer surface of the membrane relative to that at the cytoplasmic surface, Geck [4], who in dealing with the data of Lacko et al. proposed an asymmetric non-accumulative model where the inequality of the Michaelis parameters K is compensated for by a corresponding inequality of the maximal velocities and Baker & Widdas [5] who, on the basis of inhibitor studies, provided further evidence for asymmetry and developed the kinetics of a model consisting of two distinct components taking part in influx and efflux.

Paradoxically, Batt & Schachter [6] found that the affinity at the outer surface was distinctly lower than that at the inner one, and that the V/K ratio depended very much on the intracellular glucose concentration. In contrast to the above studies, Hankin et al. [7] rejected the conventional asymmetric carrier using two rigorous rejection criteria, but their experimental data have been criticised [8, 9].

In part, this paper replies to this criticism. We have here chosen to study the transport of galactose since it has lower affinity for the sugar system and therefore initial rates can be measured directly without needing an integrated rate equation treatment (and other simplifying assumptions) in the evaluation of the kinetic parameters. We have concentrated on two experimental procedures, namely measurement of the zero-trans and the infinite-cis influxes.

MATERIALS AND METHODS

Outdated heparinised blood was obtained from the blood bank. The erythrocytes were separated and washed 4 times at room temperature in buffered saline at pH 7.4 with 30 mM Tris · HCl (total osmolarity 560 mosM). The erythrocytes were kept at some 80 % hematocrit in an ice-bath prior to an experiment but never for more than 2 h.

All solutions were made up using deionized water and analytical grade reagents. The galactose incubation medium contained 30 mM Tris · HCl, pH 7.4, and aliquots of a galactose stock solution diluted serially with buffered saline to keep a constant osmolarity. When influxes were measured as a function of galactose concentration, labelled [^{3}H]galactose (The Radiochemical Centre, Amersham) at 20 mCi/mol, was added to the stock solution prior to dilution, in order to maintain a constant specific activity and as a check on the accuracy of dilution.

Fluxes were terminated at the desired times by adding a sixteenfold volume of ice-cold stopper solution: $HgCl_2$, $1 \cdot 10^{-5}$ M; phloretin (Fluka A.G.), $1 \cdot 10^{-4}$ M; KI, 1.25 mM; NaCl, 1.58 %. For radioactive counting cells were disrupted in a hemolyzing solution: $CsCl_2$, 4 mM; NH_4OH, 0.25 %; Triton X-100, 50 ppm. Scintillation liquor used was: Toluene, 66.6 % v/v; Triton X-100, 33.3 % v/v; 2,5-diphenyloxazole, 0.55 % (w/v); 1,4-bis 2-(4-methyl 5-phenyloxazolyl), 0.01 % (w/v).

"Zero-trans" influx procedure

Aliquots of 50 µl of washed erythrocytes, hematocrit approx. 80 %, were brought to 20 °C in a water bath. 0.5 ml of tritium-labelled galactose were added at $t = 0$; the reaction system was constantly mixed by means of a vortex mixer. Using a metronome as a time signal, the time of incubation with substrate could be as little as 1 s, with a reproducibility of better than 10 %. After the desired time interval, 8 ml of ice-cold stopper solution were added rapidly by means of a syringe, allowing a thorough mixing. Test-tubes containing the reaction system were centrifuged immediately in a refrigerated centrifuge (Sorvall RC-2B) at 5000 rev./min for a few seconds. The supernatant was discarded and the cells were resuspended in 8 ml of ice-cold stopper solution and spun down again. After this second wash, the cell pellet was centrifuged through a 2 cm thick layer of di-*n*-butyl phthalate in a polyethylene test-tube using the Beckman Microfuge. The test-tube tips containing the cells were cut off with a razor-blade and extracted with 3 ml of hemolyzing solution. Empty tips were

discarded and 1 ml of the extract was sampled for radioactivity measurement: to this sample was added, with vigorous stirring, 0.1 ml of cold trichloroacetic acid 100% (w/v). After 15 min of centrifugation at 5000 rev./min, 1 ml of the supernatant was transferred into vials containing 15 ml of scintillation liquor. The remaining 2 ml of hemolyzing solution was centrifuged at 15 000 rev./min for 15 min to remove the remaining phthalate which would otherwise render the solution turbid. The hemoglobin absorbance was determined photometrically at 540 nm and compared with the absorbance of a standard solution of cells, of known hematocrit, hemolyzed in a given volume of hemolyzing solution. Sampling at $t = 0$ was done by adding ice-cold substrate to ice-cold cells for 2 s and then adding stopping solution.

The galactose content of a cell suspension was computed according to the following equation:

$$C = \frac{ADP}{HS_a} \tag{1}$$

where C is the galactose content in mmol/l packed cells; A is the absorbance of 100 μl of isotonic packed cells at a hematocrit H, diluted D times in hemolyzing solution. (The ratio (AD/H) was found to be highly reproducible throughout many experiments and a mean value was therefore used with occasional checking.) P is the normalized dpm for 1 ml of hemolyzed sample:

$$P = \frac{\text{cpm of sample} - \text{background}}{\text{efficiency} \times \text{absorbance of sample}} \tag{2}$$

S_a is the specific activity of the galactose in the incubation medium. The galactose concentration in units of cell water was calculated using the cell water to packed cell ratio, obtained in a separate experiment by determining fresh and dry weights of blood samples at given osmolarities of external medium and known hematocrit.

"Infinite-cis" influxes

Prewashed cells (see Results section) were equilibrated with different concentrations of [^{3}H]galactose at 1 : 10 v/v ratio. After the periods of time required for equilibration, the cells were centrifuged at 7000 rev./min for 2 min and the supernatant was discarded. Labelled galactose at exactly the same specific activity as that used in the initial loading step but at 500 mM, was then added. The uptake of sugar was allowed to proceed for various periods of time (the time was adjusted according to the concentration of the preloading solution so as to obtain in each case significant increments of galactose content). Stopping, washing-out the trapped extracellular sugar and galactose determination were carried out as described above. The controls were incubated with 560 mosM buffered saline free of galactose for the average preloading time.

Enzymatic determination of intracellular glucose

Intracellular glucose was determined by analysing the hemolysate, using an enzymatic colorimetric method modified according to Raabo & Terkildsen [10]. This method is based on simultaneous use of glucose oxidase and peroxidase coupled with the chromogenic oxygen acceptor *O*-dianisidine dihydrochloride. All materials

were purchased from Sigma Chemical Co. and the procedure was according to Sigma's technical bulletin No. 510.

RESULTS

Effectiveness of stopping and washing procedures

A major difficulty in the accurate assay of low levels of intracellular galactose is the substantial contribution that the extracellular space makes to the total galactose content of the cell sample. According to the washing procedure described in the Methods section, each washing dilutes the extracellular space about 80 fold (16-fold dilution where extracellular is 1/5 of the total). With 500 mM in the incubation medium and an expected intracellular concentration of, say, 1 mM, at least three washings are required in order to reduce the extracellular activity below 10 % of the intracellular one. Obviously, several consecutive washings bring about an inevitable loss of cells. Moreover, the stopping technique is effective for only a limited period of time [11]. By using di-*n*-butyl phthalate (spec. gravity 1.042–1.049) it is possible to separate the erythrocytes from their bathing medium, thus reducing the number of washings to 2 only and the overall time needed for the complete procedure, from stopping to hemolysis, to about 5 min for a group of 8 samples.

To test the efficiency of the stopping procedure, washed cells were equilibrated with 1 and 125 mM labelled galactose at 20 °C. Some samples were spun directly through phthalate and then hemolyzed. Others were first mixed with stopper solution, spun down and then hemolyzed. Other samples were washed twice or three times prior to hemolysis (see Table I). Controls at $t = 0$ for the determination of radioactivity carry-over and non-specific adsorption to cells and test-tube walls, were run simultaneously. The controls for background levels were obtained by mixing precooled cells with ice-cold substrate for 2 s followed by two subsequent washings with ice-cold stopping solution. Results are presented in Table I.

Obviously, direct centrifugation of the cell suspension through the phthalate is inadequate in cases of high total radioactivity, since the cells apparently carry over, non-specifically, relatively large amounts of the label. However it is evident that our stopping procedure is effective in preventing large leaks from the cells during the centrifugation and resuspension steps.

TABLE I

Effectiveness of stopping and washing procedure. Cells were equilibrated with either 1 or 125 mM galactose. Samples were then centrifuged through phthalate (no wash). Other samples were first mixed with cold stopper, then spun down and the resulting pellet was then centrifuged through phthalate (1 wash). Additional samples were further washed two or three times as indicated (2 washes, 3 washes). The concentration of sugar entering the cells in a 2 s loading period at 0 °C were obtained as detailed in the text (2 s entry).

Galactose concn (mM)	mmol/l packed cells					Galactose concn (mmol/l cell H_2O) (2 washes)
	No wash	1 wash	2 washes	3 washes	2 s entry	
1	0.462 ±0.008	0.450±0.012	0.430±0.030	0.442±0.002	0.00	0.88±0.06
125	100.75±4.37	70.50±0.82	69.45±0.62	65.66±2.28	0.32	126.1±1.1

Importance of prewashing the erythrocytes

A zero-trans influx experiment is very sensitive to traces of intracellular glucose. Since anticoagulant media for blood preservation used currently in blood banks contain a relatively high glucose concentration (25 mM), the prewashing of cells for a zero-trans experiment should be very meticulous. In order to test the effect of residual internal glucose, simultaneous measurements of intracellular glucose by the enzymatic-colorimetric method and of the influx of [^{3}H]galactose were carried out. Results are presented in Fig. 1. It is natural to suspect that such a system might obey a relation of the form

$$v = \left(1 + \frac{S_i \cdot \alpha}{K + S_i}\right) V \tag{3}$$

where v is the measured rate of galactose uptake; V is the initial rate of galactose uptake in the absence of intracellular glucose; αV that in the presence of limitingly high intracellular glucose; S_i is the concentration of intracellular glucose in mM; K is a half-saturation concentration for glucose at the inner side of the membrane. On transforming this equation into:

$$\frac{V}{v-V} = \frac{1}{\alpha} + \frac{K}{\alpha} \cdot \frac{1}{S_i} \tag{4}$$

a graph of $V/(v-V)$ vs $1/S_i$ gives both α and K. This has been done for the present case giving $\alpha = 5.7$; $K = 7.0$ mM. Interestingly, K is intermediate in value between

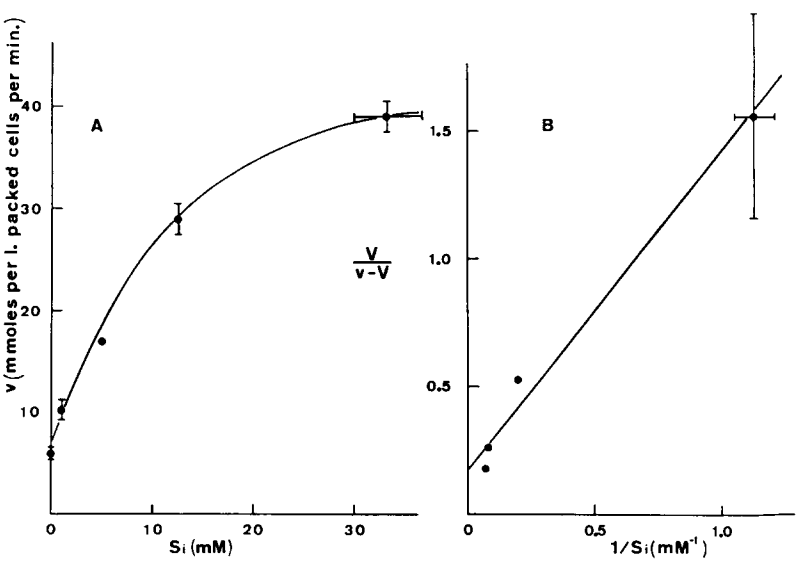

Fig. 1. Uptake of galactose in the presence of internal glucose. Cells were partially washed leaving some of the glucose remaining inside, and then mixed with 31.25 mM galactose in buffered saline, pH 7.4, at 20 °C for short periods of time (1–5 s) and then washed and counted as described in Methods. Cellular glucose was measured simultaneously on parallel samples by an enzymatic method. (A) Initial rate of galactose uptake vs intracellular glucose concentration. (B) Double reciprocal plot of the same data.

the low affinity site of 25 mM reported by Karlish et al. [11] and the high affinity site of 2.8 mM reported by Hankin et al. [7]. Indeed from Fig. 1b it might appar that the dependence of $V/(v-V)$ on $1/S_i$ could be better fitted by two straight lines. An important conclusion from Fig. 1 is that glucose and galactose use the same transport system. Incidentally, zero-trans influx is the only mode of measurement of transport which is sensitive to preliminary washing, since in the other modes, i.e. zero-trans efflux [11] and equilibrium exchange [14], there is a step of preincubation during which glucose can leave the cells or at least decrease in concentration to an insignificant level.

The time course for galactose uptake

The time course of galactose influx can be studied from two aspects. One can measure initial uptake rates (where insignificant amounts of galactose have entered the cell) and hence obtain the K_m and V of the transport system under zero-trans conditions. However, when in such experiments at high external substrate concentration the cells begin to accumulate substrate, the zero-trans conditions transform into an infinite-cis situation from which the K_m of the intracellular substrate-binding site (K^{ic} = "Sen-Widdas" K_m) can be calculated. In both cases the existence of more than one site may be detectable.

Fig. 2 represents a typical time course of galactose uptake from a concentration of 250 mM galactose in the external medium. From the inset it is obvious that influx is linear up to 10 s, deviating thereafter. A zero-trans flux can be calculated from the

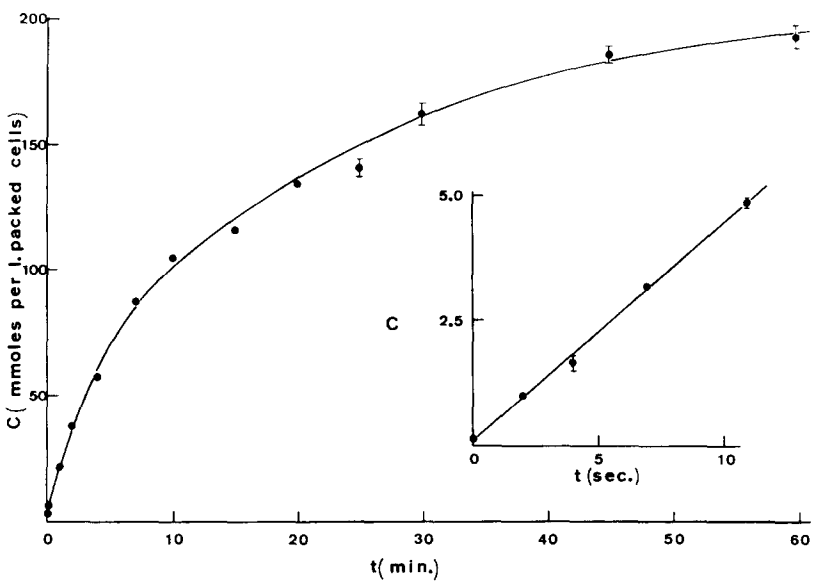

Fig. 2. Uptake of galactose as a function of time. Washed cells were mixed with a solution of 250 mM labelled galactose in buffered saline, pH 7.4, having total osmolarity of 560 mosMol/l at 20 °C. After various time intervals, uptake was stopped and measured as detailed in Methods. Values are given as mean ± S.E. of 4 samples. The curve drawn has no theoretical meaning. The uptake at short periods is given in the inset, the straight line being drawn by a regression analysis.

slope of data obtained at short loading times. The intercepts on the Y-axis obtained by regression analysis differed insignificantly from the relevant $t = 0$ controls. On the basis of these findings, then, when in subsequent experiments zero-trans influx was studied as a function of the external galactose concentration a period of 5 s was allowed for uptake.

If the whole time course is considered, additional parameters of the transport system can be obtained. Eilam and Stein [12] have generalised the kinetic treatment of Lacko et al. [3] for the case of asymmetric carrier. If one starts from their Eqn (70) taking the case where the internal substrate concentration is initially 0, one gets

$$(A+BS_0)\ln\frac{S_0-C}{S_0}+B=-t \tag{5}$$

where $A = KR_{00}+R_{21}S_0$ (6)

$$B=\frac{KR_{00}}{\pi}+R_{12}+\left(\frac{R_{12}}{\pi}+\frac{R_{21}}{\pi}+\frac{R_{ee}}{K}\right)S_0+\frac{R_{ee}}{K\pi}S_0^2 \tag{7}$$

π is the concentration of osmotically active but impermeable solute, S_0 is the external substrate concentration, C is the internal substrate concentration, R_{ee} is the reciprocal of the maximum velocity per unit carrier for the equilibrium-exchange experiment, R_{12} is the reciprocal of the maximum velocity per unit carrier of the zero-trans experiment in the $1 \rightarrow 2$ direction, R_{21} is the corresponding quantity measured in the $2 \rightarrow 1$ direction, $R_{00} = R_{12}+R_{21}-R_{ee}$ and K is the half saturation concentration. These parameters are further discussed in Eilam and Stein's paper [12]. Algebraic transformation of Eqn (5) yields:

$$(A+BS_0)\left[\frac{\ln\left(1-\frac{C}{S_0}\right)+\frac{C}{S_0}}{C}\right]-\frac{A}{S_0}-=\frac{t}{C} \tag{8}$$

It is now possible to plot t/C against $[\ln(1-C/S_0)+C/S_0]/C$ and to get

$$Y_{-\text{cept}} = A/S_0 = \frac{KR_{00}}{S_0}+R_{21} \tag{9}$$

since $X_{-\text{cept}} = \dfrac{A}{S_0(A+BS_0)}$, then $S_0^2 X_{-\text{cept}} = \dfrac{AS_0}{A+BS_0}$.

At limitingly high substrate concentration (with respect to K) one gets

$$\lim_{S_0\rightarrow\infty} S_0^2 X_{-\text{cept}} = \frac{A}{B} = \frac{KR_{21}\pi}{R_{ee}S_0} = \frac{\pi}{S_0}K^{ic} \tag{10}$$

Fig. 3. demonstrates results obtained from the time course of uptake of galactose at 250 mM. The regression analysis of this line yields an $X_{-\text{cept}} = 4.24 \cdot 10^{-4}$ with a correlation coefficient of 0.96 $K^{ic} = 250^2 \cdot 4.24 \cdot 10^{-4} \frac{250}{310} = 21.4$ mM. The standard error of the mean is 16.6. The value of K^{ic} is that cellular galactose concentration which reduces the rate of uptake to one half of the initial rate.

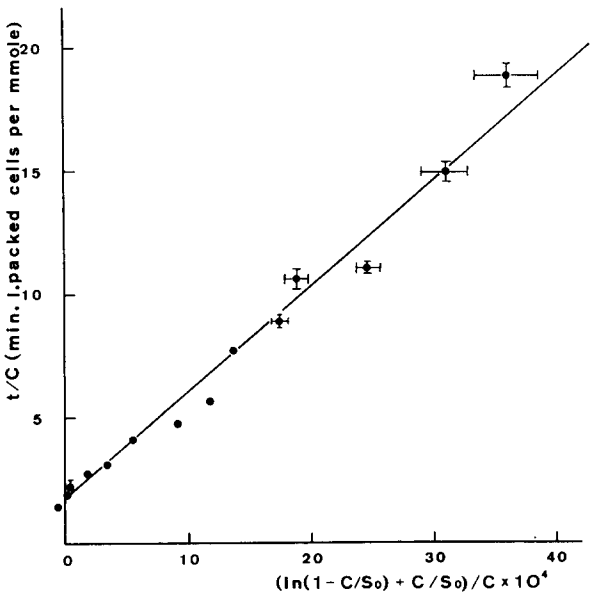

Fig. 3. Double reciprocal plot using the integrated rate equation of the data presented in Fig. 2. The regression analysis of the plot yields the following values: slope = 4219±806; Y_{-cept} = 1.79±1.42; correlation coefficient, r = 0.961. From these values one gets K^{ic} = 21.4±16.6 mM.

"Infinite cis" influx

This procedure is based on that introduced (for efflux) by Sen and Widdas [13]. In the present work "infinite cis" influx experiments were performed by preloading the cells to various concentrations of tritiated galactose at 20 °C. Cells were then spun down and redispersed in 500 mM tritiated galactose, of exactly the same specific activity as that used for preloading, for various periods of time for the second uptake step. Stopping and washing procedures were as usual. The rate of influx was plotted against that intracellular concentration measured during the middle of the second uptake period. Intracellular concentration in terms of cell water was calculated from the substrate content C measured in cell units according to the following equation:

$$S_i = \frac{C}{0.5 + bC} \tag{11}$$

where S_i is the concentration in mM, 0.5 is the relative cellular water content at 560 mosM in the bathing medium and b is the slope of an experimentally determined calibration curve relating the water content to the osmolarity of the medium. This calibration curve was obtained by suspending cells in saline solutions of different osmolarities and determining the water content in the usual way. The term $(0.5+bC)$ gives the cellular water content when the osmotic pressure difference is being reduced by the entry of substrate to an intracellular concentration C. Fig. 4 shows a representative experiment of the time dependence of uptake by loaded cells. Fig. 5 shows a replot of the data of Fig. 4 where we have plotted $1/V_s$ against S_i, S_i being calculated

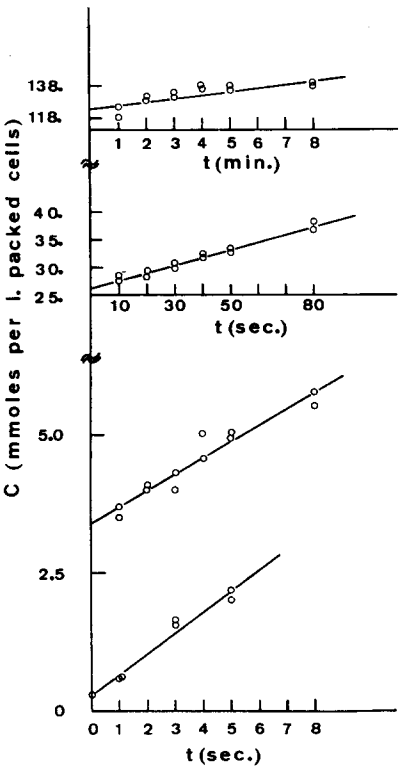

Fig. 4. Uptake of galactose into cells preloaded to different intracellular galactose concentrations, as a function of time. Washed cells were equilibrated at 20 °C with radioactive galactose for 1 h. Cells were then separated from the equilibration medium and mixed with a solution of buffered galactose at 500 mM having the same specific activity as the equilibration solutions, pH 7.4, 20 °C. After various periods of time uptake was stopped and measured as detailed in Methods. Note the different time scales and the discontinuity of the abscissa. Curves were obtained by linear regression.

as above, V_s being the slope of the uptake with time. The mean value for the parameter K^{ic} derived from 5 such experiments, 20 slopes in all, is 24.9 ± 17.6 mM. Thus we see that with 2 different experimental procedures, the same values of K^{ic} are obtained.

"Zero-trans" influx as a function of galactose concentration

The "zero-trans" influx as a function of galactose concentration was studied by incubating aliquots of cells for 5 s to a range of substrate concentrations from 1 to 500 mM. Stopping and washing procedures were done as usual and each concentration was assayed in quadruplicate. Fig. 6 demonstrates a typical experiment. The analysis of data can be done in several ways. Here, a non-linear least square fit analysis of v against S_0 was carried out by means of the maximum neighborhood method, using library program NONLSQ of the Hebrew University Computation Centre. Thus several methods of transport as follows could be tested:

1) One specific carrier
2) One specific carrier in parallel with a leak
3) Two specific carriers in parallel

Although the standard errors increase upon the addition of parameters, it is possible to check the improvement in fitting, by means of an F-test, using the following equation:

$$F_x = \frac{\chi^2(n-1)-\chi^2(n)}{\chi^2(n)/(N-n-1)} \tag{12}$$

where χ^2 (n) is the chi-square associated with the deviations about the regression with n parameters. N is the number of points used in the analysis. Considering the F values in Table II it is clear that the 3-parameter model is statistically an improvement over the 2-parameter model. The improvement upon the addition of the fourth parameter is not statistically significant. We show in the next section how the choice between the two latter alternatives can be tested experimentally; the non-specific uptake should not be inhibited by specific inhibitors such as phloretin.

TABLE II

Results of non-linear least square fit analysis of initial uptake rate vs substrate concentration using different models. Parameters ± S.E. were derived using NONLSQ library program of the Hebrew University Computation Centre. F_x was calculated from Eqn (12). The units for the V values and a are given in mmol/cell unit per min. The K values are given in mM.

Model	Derived parameters ± S.E. ($N = 76$)				F_x
1) $\frac{VS_0}{K+S_0}$	$V =$ 28.6 ± 1.12	$K =$ 31.76 ± 4.42			
2) $\frac{VS_0}{K+S_0}+aS_0$	$V =$ 21.37 ± 1.93	$K =$ 17.4 ± 4.01	$a =$ 0.018 ± 0.005		9.76 $P < 0.005$
3) $\frac{V_1S_0}{K_1+S_0}+\frac{V_2S_0}{K_2+S_0}$	$V_1 =$ 15.86 ± 6.01	$K_1 =$ 11.21 ± 6.40	$V_2 =$ 21.47 ± 15.83	$K_2 =$ 285.9 ± 282.1	1.53 $P \approx 0.2$

Inhibition of galactose transport by phloretin

Since phloretin is a powerful competitive inhibitor of the hexose transport system, it can be used to decide whether simple diffusion plays a significant part in the galactose uptake that we have measured. In order to obtain the inhibition constant (K_I) for the action of phloretin on sugar entry, we measured uptake as a function of phloretin concentration at 8 mM galactose where the contribution of simple diffusion is insignificant (less than 1.5 % using the parameters of model 2 from Table II). From simple kinetic considerations the ratio of inhibited (v_i) to uninhibited (v_u) uptake is given by

$$\frac{v_i}{v_u} = \frac{K+S_0}{K+S_0+\frac{K \cdot I}{K_I}} \tag{13}$$

where S_0 is the concentration of substrate, I that of inhibitor, K is the Michaelis constant for the substrate. From the experimental data (not shown), 50 % inhibition is found by interpolation, to be when $I = 5 \cdot 10^{-6}$ M. Taking $K = 17$ mM from Table

II, K_I is found to be $3.4 \cdot 10^{-6}$ M. (LeFevre [15] found K_I to be $4.4 \cdot 10^{-6}$ M for inhibition of glucose efflux at 37 °C.)

Using this parameter, one can now predict the relative inhibition when the substrate concentration is 500 mM on two models: (1) taking into account that a significant portion (30 %) (see Table II) may be entering by simple diffusion and (2) the model of two carriers in parallel, using parameters of Table II. The experimental data (not shown) do not allow a choice between the two models. A distinction can, however, be made as follows: The cells are preloaded to a certain concentration of galactose which, as we have shown already, brings about a reduction of net transport. The residual transport will be further inhibited by phloretin if it is carrier mediated. The results of such an experiment are shown in Table III. On the assumption that a simple diffusion component is present with the values derived in Table II, and that net transport in such a system is reduced by 62.5 mM inside according to Fick's law, the residual uptake after inhibition of the facilitated diffusion by Phloretin in this system would be 27 % of the control: very significantly different from 4.1 ± 3.6 % found experimentally. Thus the combination of preloading and phloretin inhibition leads us to reject the model that simple diffusion plays a significant part in the transport.

TABLE III

Phloretin inhibition of galactose uptake into cells preloaded to 62.5 mM. Results are given as percentage of control, i.e. neither phloretin nor internal galactose is present.

Phloretin concentration (mM)	Galactose uptake (% of control)
0	26.9 ± 4.3
$1 \cdot 10^{-5}$	29.1 ± 6.1
$1 \cdot 10^{-4}$	11.6 ± 5.0
$1 \cdot 10^{-3}$	4.1 ± 3.6

DISCUSSION

As described above the analysis of the trans-zero uptake experiments enabled us to reject on statistical grounds the model of a single carrier. But we have felt it desirable to show in an independent way that this conclusion is justified. To do this we have devised an analytical procedure which one might call the "cis/trans test". In this test one makes use of 3 experimental measurements namely zero-trans uptake rates at 2 concentrations, S_1 and S_2, namely $v_{s_1}^{zt}$ and $v_{s_2}^{zt}$ respectively, where $S_1 \gg K_m$ and $S_2 < S_1$ and the net uptake with S_1 at the cis and S_2 at the trans side of the membrane, v_{S_1/S_2}^{ic}. The ratio $r = (v_{s_1}^{zt} - v_{S_1/S_2}^{ic})/v_{s_2}^{zt}$ is an indication of the directionality of the transport system (see appendix) but its dependence on S_2 allows us to distinguish between the different models concerned.

In Fig. 7 we have computed the values of r as a function of S_2, the concentration at the trans face, for the three models of Table II using the respective zero-trans parameters listed in that table and the infinite-cis parameter derived in Fig. 5. Experi-

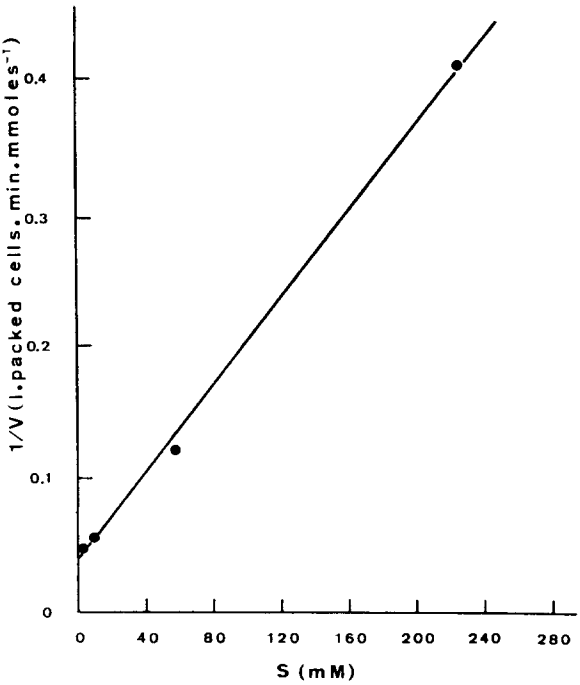

Fig. 5. Reciprocal uptake rate as a function of intracellular galactose. Data obtained from Fig. 4.

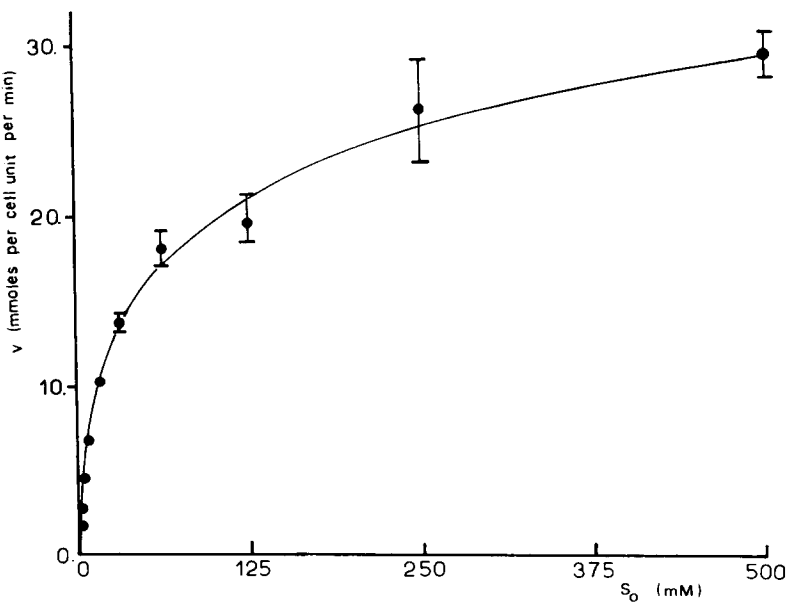

Fig. 6. Galactose uptake rate as a function of galactose concentration in the medium. Washed cells were exposed to various concentrations of radioactive galactose in buffered saline. Total osmolarity 560 mosmol/l, pH 7.4, 20 °C. Uptake was stopped after 5 s and measured as detailed in Methods. Mean values are given ±standard errors.

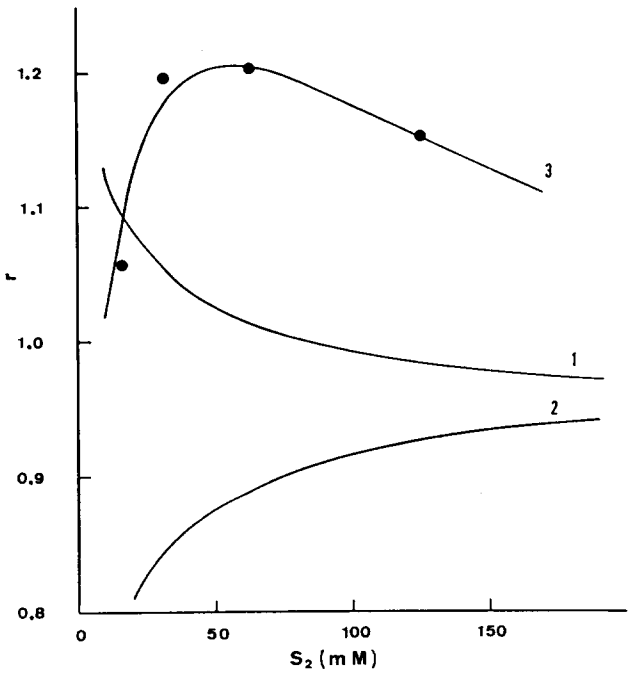

Fig. 7. Cis-trans test curves for various transport models and experimental values. Curve 1 describes a one-site model. Curve 2 describes a one-site model in parallel with simple diffusion. Curve 3 describes a two-site model. Curves were computed using the respective parameters of Table II and the infinite-cis K derived in Fig. 5. Experimental values (●) were taken directly from Figs 5 and 6.

mental values for the fluxes used to obtain the experimental values of r, were taken directly from Figs 5 and 6.

The one-carrier model gives a predicted curve which is totally different from experimental observations allowing the rejection of this model. The one carrier-carrier+simple diffusion model gives, always, values which are smaller than 1, while the experimental observations are always significantly greater than 1 and are in good agreement with the predicted curve for the two-carrier model. It should be emphasized that the predicted curves were computed from zero-trans parameters using a common value for the infinite-cis K_m inside. No attempt has been made to fit the theoretical curves to the experimental data of Fig. 7.

The results described in Table III of the effect of phloretin inhibition and galactose preloading, provide strong and independent support for the view that simple diffusion plays a minor part in galactose influx. It is difficult to escape the conclusion that two parallel sites mediate the transport of galactose into human erythrocytes.

It is possible to understand why Lacko et al. [3] failed to see a low affinity site for glucose entry into human red cells: in the first place they did not study sufficiently high concentrations of sugar, but more important their sampling times, 4 s minimum, were too long for the evaluation of true initial rates. The infinite cis K_m for glucose at the inner face of the membrane will be some 2–3 mM (Hankin et al. [7]).

As far as events occurring at the inner face of the membrane, our results show clearly a high affinity site for galactose (high, that is, compared with the value of

140 mM for equilibrium exchange [14]. Such a site has been proposed for glucose by Hankin et al. [7], although a doubt has been raised as to the validity of these results (Regen & Tarpley [8]). The latter authors' remark that "a modest error in washing out or subtracting extracellular glucose would affect the conclusions seriously" cannot apply to the present study where extremely careful precautions have been used to reduce the extracellular galactose. Regen & Tarpley also "Question" the "incompatible time course" of the Hankin et al. experiment. The preloading experiments provide direct evidence, using initial rates only, for the effect of internal galactose on galactose entry and they are not subject to this kind of criticism.

On the basis of the experiments of Karlish et al. [11] on glucose efflux which demonstrated a low affinity site for glucose at the inner face of the membrane, we expected to see an analogous site for galactose to be reflected in the infinite-cis K_m. However, although we went up to 300 mM we could not detect such a site. Either it does not exist or other methods are required for its identification.

Our results indicate that at the outer face of the erythrocyte membrane there exist a high and a low affinity site for galactose; at the inner face we have identified a high affinity site. What model for transport will turn out to be compatible with these results is left for further study.

APPENDIX

Taking first the case of a simple diffusion system one can define a quantity

$$r = \frac{v_{s_1}^{zt} - v_{s_1/s_2}^{ic}}{v_{s_2}^{zt}} = \frac{aS_1 - a(S_1 - S_2)}{aS_2} = 1 \tag{A1}$$

with a as the permeability coefficient.

Thus, for this case, r is identically 1 for all values of S. It has previously been shown [16] that the unidirectional flux of substrate for a generalized asymmetric carrier can be written as:

$$\frac{U_{12}}{T} = \frac{KS_1 + S_1 S_2}{K^2 R_{00} + KR_{12} S_1 + KR_{21} S_2 + R_{ee} S_1 S_2} \tag{A2}$$

where the parameters K and R are defined in that paper; U_{12}/T is the unidirectional flux per unit carrier. For our purposes let S_1 and S_2 be the outside and inside galactose concentrations respectively. For the net influx the numerator of Eqn (A2) is replaced by $K(S_1 - S_2)$. We can rearrange the denominator as:

$$\text{Den.} = \left[\frac{KR_{12}}{R_{ee}}\left(\frac{KR_{00}}{R_{12}} + S_1\right) + \left(\frac{KR_{21}}{E_{ee}} + S_1\right) S_2\right] R_{ee} \tag{A3}$$

We make the simplifying assumptions now that when S_1 is sufficiently high in comparison with both KR_{00}/R_{12} $(= K_{12}^{zt})$ and KR_{21}/R_{ee} $(= K_{21}^{ic})$, then

$$\left(\frac{KR_{00}}{R_{12}} + S_1\right) \approx \left(\frac{KR_{21}}{R_{ee}} + S_1\right)$$

With this assumption the denominator will be

$$\text{Den.} = (K_{12}^{zt}+S_1)(K_{12}^{ic}+S_2)R_{ee} \tag{A4}$$

thus for any carrier

$$r = \frac{v_{s_1}^{zt} - v_{s_1/s_2}^{ic}}{v_{s_2}^{zt}} = \frac{\dfrac{S_1 V_{12}^{zt}}{K_{12}^{zt}+S_1}\left(1 - \dfrac{K_{12}^{ic}}{K_{12}^{ic}+S_2}\right)}{\dfrac{S_2 V_{12}^{zt}}{K_{12}^{zt}+S_2}} = \frac{S_1(K_{12}^{zt}+S_2)}{(K_{12}^{zt}+S_1)(K_{12}^{ic}+S_2)} \tag{A5}$$

In passing we note that when S_1 is large $r = (K_{12}^{zt}+S_2)/(K_{12}^{ic}+S_2)$. As S_2 increases r approaches unity (the value obtained for simple diffusion), but does so from above or below according as K_{12}^{zt} is greater than or lesser than K_{12}^{ic}. Thus for an asymmetric carrier with the high affinity site facing outwards, approach to unity will be from below and vice versa for the oppositely directed carrier.

For the case where we have simple diffusion in parallel with the carrier, we need merely add the quantity aS_2 to both numerator and denominator:

$$r = \frac{\dfrac{S_1 S_2 V_{12}^{zt}}{(K_{12}^{zt}+S_1)(K_{12}^{ic}+S_2)} + aS_2}{\dfrac{S_2 V_{12}^{zt}}{K_{12}^{zt}+S_2} + aS_2} \tag{A6}$$

And for the model of two carriers in parallel the numerator and denominator contain terms from both carriers.

ACKNOWLEDGEMENTS

We wish to thank Ms Y. Baumgarten for technical assistance of a high order, Drs Y. Eilam and W. R. Lieb for helpful discussions and Ms A. Tolkovsky for help in the statistical evaluation of the data. This work was supported by the Professor Philip Stein Fellowship Fund, generously sponsored by Sagov Industrial Management Co. Ltd. of South Africa.

REFERENCES

1 Le Fevre, P. G. (1974) Current Topics in Membrane Transport (Bonner, F. and Kleinzeller, A., eds), Academic Press, New York, in the press
2 Lieb, W. R. and Stein, W. D. (1972) Biochim. Biophys. Acta 265, 187–207
3 Lacko, L., Wittke, B. and Kromphardt, H. (1972) Eur. J. Biochem. 25, 447–454
4 Geck, P. (1971) Biochim. Biophys. Acta 241, 462–472
5 Baker, G. F. and Widdas, W. F. (1973) J. Physiol. 231, 143–165
6 Batt, E. R. and Schachter, D. (1973) J. Clin. Invest. 52, 1686–1697
7 Hankin, B. L., Lieb, W. R. and Stein, W. D. (1972) Biochim. Biophys. Acta 288, 114–126
8 Regen, D. M. and Tarpley, H. L. (1974) Biochim. Biophys. Acta 339, 218–233
9 Edwards, P. A. W. (1973) Biochim. Biophys. Acta 311, 123–140
10 Raabo, E. and Terkildsen, T. C. (1960) J. Clin. Lab. Invest. 12, 402–407
11 Karlish, S. J. D., Lieb, W. R., Ram, D. and Stein, W. D. (1972) Biochim. Biophys. Acta 255, 126–132

12 Eilam, Y. and Stein, W. D. (1974) Methods in Membrane Biology (Korn, E. D., ed.), Plenum Press, New York, in the press
13 Sen, A. K. and Widdas, W. F. (1972) J. Physiol. London 160, 392–403
14 Eilam, Y. and Stein, W. D. (1972) Biochim. Biophys. Acta 266, 161–173
15 Le Fevre, P. G. (1954) S.E.B. Symposium: Active Transport and Secretion (Brown, R. and Danielli, J. F., eds), Cambridge University Press, Cambridge
16 Stein, W. D. and Lieb, W. R. (1973) Israel J. Chem. 11, 325–339