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ZERO-TRANS AND EQUILIBRIUM-EXCHANGE EFFLUX AND INFINITE-TRANS UPTAKE OF GALACTOSE BY HUMAN ERYTHROCYTES

HAGAI GINSBURG and DALIA RAM

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

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SUMMARY

1. The zero-trans and equilibrium exchange efflux and the infinite-trans uptake of galactose in human erythrocytes were measured as a function of galactose concentration at 20 °.

2. The zero-trans procedure with cells loaded with 285 mM galactose revealed a low affinity site for galactose transport at the inner face of the membrane having a maximal velocity of 255 ± 96 mmol/l isotonic cell water and $K_m = 240 \pm 57$, the V/K ratio being 1.01 ± 0.04 min^{-1}.

3. The equilibrium-exchange procedure yielded a maximal velocity of 432 ± 44 mmol/cell unit per min and $K = 138 \pm 57$, the V/K ratio being 3.19 ± 0.52 min^{-1}.

4. The infinite-trans uptake revealed a high affinity site at the outer face of the membrane having a maximal velocity of 239 ± 11 mmol/cell unit per min, and $K = 21 \pm 2$ mM.

5. These results combined with previous findings (Ginsburg, H. and Stein, W. D. (1975) Biochim. Biophys. Acta 000, 000–000) force us to reject the following models for sugar transport in human erythrocytes: a single asymmetric carrier; two symmetric carriers in parallel, the original form of the internal transfer model.

INTRODUCTION

In the preceding [1] we have demonstrated the existence of two sites for galactose uptake in human erythrocytes, one of higher and one of lower affinity for the substrate. We could also demonstrate the occurrence of a high-affinity site at the inner side of the membrane.

In order to widen the experimental base for the theoretical analysis we have in the present work studied additional experimental procedures, namely zero-trans efflux, equilibrium exchange and infinite-trans influx. Each of these procedures bears on possible models for sugar transport, as will be seen in the discussion.

MATERIALS AND METHODS

The following solutions were used for the equilibrium exchange and the zero-trans efflux:

(1) Buffer: NaCl, 147 mM; Na_2HPO_4, 20 mM. pH was adjusted to 7.4 with HCl. Total osmolarity was 310 mosmol/l.
(2) Stopper solution: NaCl, 1 % (w/v); $HgCl_2$, 10^{-6} M; KI, 1.25 mM; Phloretin (dissolved in methanol), $1 \cdot 10^{-4}$M and 1 % ethanol.
(3) Scintillation liquid: toluene, 68 % (v/v); ethanol, 32 % (v/v); 2,5-diphenyloxazole (PPO), 0.4 % (w/v); 1,4-bis-2-(4 methyl-5-phenyloxazolyl)-benzene (POPOP), 0.04 % (w/v). Alternatively, a toluene triton X-100 2:1 v/v solution containing 0.55 % PPO and 0.01 % POPOP was used.

Materials used were Analar phloretin from Fluka A.G. and D-[^{14}C] galactose from the Radiochemical Centre.

Zero-trans efflux procedure

This procedure was almost indentical to that used by Karlish et al. [2]. Erythrocytes obtained from outdated transfusion blood were washed 3 times in the NaCl/Na_2HPO_4 buffer and then preloaded with galactose to a final concentration of 285 mM, at 5 % hematocrit and 37 °C. Thereafter radioactive galactose was added and equilibration was allowed to proceed to completion. To follow the time course of galactose efflux 0.2 ml of preloaded cells were poured rapidly with vigorous stirring into 100 ml of the NaCl/Na_2HPO_4 buffer with the addition of sufficient NaCl to maintain isotonicity. At given times aliquots of this mixture were mixed with ice-cold stopper solution at a ratio of 1:4 (v/v). Cells were then spun down and the pellet was resuspended in the buffer. Samples were mixed vigorously with 20 % trichloroacetic acid and centrifuged. Aliquots from the clear supernatant were mixed into scintillation liquor for radioactive counting. The relative number of cells in each pellet was determined by measurement of the hemoglobin content using the standard Drabkin method, and radioactive content of the cells at $t = 0$ and $t = \infty$ were also determined.

Equilibrium exchange procedure

This procedure was very similar to that used by Eilam & Stein [3]. Erythrocytes from outdated transfusion blood were washed in NaCl/Na_2HPO_4 buffer and then equilibrated with the required concentration of galactose in the buffer for 1 h at 37 °C. Radioactive galactose was then added and equilibration was allowed to proceed to completion. Cells were then mixed with the buffer containing galactose at a concentration equal to that of the loading solution. At the appropriate time samples of the mixture were mixed with ice-cold stopper solution and centrifuged. Controls of $t = 0$ and $t = \infty$ were done simultaneously. The determination of radioactivity and relative number of cells in the sample were done as in the zero-trans procedure.

Infinite-trans procedure

Cells from outdated transfusion blood were washed in NaCl buffered with Tris · HCl, pH 7.4, at a total osmolarity of 560 mosmol/l at room temperature. Cells were then equilibrated in a medium containing 136 mM NaCl, 250 mM galactose and 30 mM Tris · HCl for 2.5 h at 37 °C. Cells were then spun down and as much as possible of the supernatant was discarded. The Hematocrit of the remaining pellet was measured. 50 µl of cells preequilibrated to 20 °C were mixed vigorously with 0.5 ml of labelled galactose solution. Uptake was stopped after the required period of time (1, 3, 5, 10 s) by adding 8 ml stopper solution. Cells were then treated as described

by Ginsburg & Stein [1]: they were centrifuged and resuspended in new stopper solution, centrifuged again and then 100 μl of the pellet were centrifuged across a 2 cm thick layer of di-*n*-butylphthalate. The pellet was mixed in hemolyzing solution ($CsCl_2$, 4 mM; NH_4OH, 0.25 %; Triton X-100, 50 ppm) from which samples were taken for counting radioactivity and the hemoglobin absorbance.

RESULTS

Zero-trans efflux

Experimental data were analyzed by a method modified slightly from that used by Karlish et al. [2]. Starting from their Eqn (2) it can be readily shown that

$$\frac{t}{1-f} = \frac{KP}{V(P+S_0)} \frac{\ln f}{1-f} + \frac{K+P+S_0}{P+S_0} \cdot \frac{N_0}{V} \tag{1}$$

where t is time of sampling, f is the fraction of galactose remaining in the cells at time t, K is the Michaelis constant, V is the maximal rate of uptake, S_0 is the external galactose concentration, P is the concentration of internal impermeable+osmotically active material and N_0 is the internal concentration of galactose at true zero, corrected for volume increase. A plot of $t(1-f)$ vs $\ln f/(1-f)$ should give a straight line with intercept A and slope B from which we can derive the following parameters

$$V/K = \frac{P}{P+S_0} \cdot \frac{1}{B} \tag{2}$$

$$K = \frac{P+S_0}{\frac{PA}{N_0 B} - 1} \tag{3}$$

$$V = \frac{KP}{B(P+S_0)} \tag{4}$$

Regression analysis was done on data from 4 experiments and results are presented in Table I. The weighted mean values $\pm$standard errors are: $V = 254.7 \pm 96.1$

TABLE I

Results of zero-trans efflux experiments. Results are given as mean $\pm$S.E. B is the slope of the straight line relating $t/(1-f)$ to $\ln f/(1-f)$ obtained by linear regression, A is the intercept of the same line and r is the correlation coefficient.

Experiment No.	B	A	r	V/K (min^{-1})	K (mM)	V (mmol/l isotonic cell water per min)
1	27.48 $\pm$ 1.64	73.9 $\pm$ 3.53	0.985	1.14 $\pm$ 0.07	311.5 $\pm$ 156.6	355.0 $\pm$ 266.7
2	25.07 $\pm$ 1.95	87.6 $\pm$ 3.36	0.970	1.25 $\pm$ 0.10	212.5 $\pm$ 97.9	265.6 $\pm$ 195.3
3	23.98 $\pm$ 1.96	77.84 $\pm$ 4.40	0.975	1.30 $\pm$ 0.11	235.2 $\pm$ 122.7	305.8 $\pm$ 238.1
4	37.67 $\pm$ 2.23	118.76 $\pm$ 3.45	0.960	0.83 $\pm$ 0.05	244.9 $\pm$ 102.8	203.3 $\pm$ 140.8
Weighted Average $\pm$ S.E.				1.01 $\pm$ 0.04	240.6 $\pm$ 57.1	254.7 $\pm$ 96.1

mmol/min per l isotonic cell water. $K = 240.6 \pm 57.1$ mM and $V/K = 1.01 \pm 0.04$. This demonstrates clearly a low affinity site for galactose transport at the inner face of the membrane.

Equilibrium exchange

For the analysis of the data of this experimental procedure we followed Eilam & Stein [3]. We used their equation

$$\ln \frac{Ct}{C_0} = \frac{-V}{K+S} t \tag{5}$$

where Ct and C_0 are the radioactivities present in the cells at times t and 0 respectively, V is the maximal velocity of exchange, K is the Michaelis constant, S is the concentration of the galactose used. We carried out a regression analysis of $\ln C_t$ vs t for each concentration of galactose ranging from 50 mM to 400 mM. Then the reciprocal of the slope which is equal to $(K+S)/V$ was plotted against S and the regression analysis yielded then a slope which is equal to $1/V$ and an intercept equal to K/V. Results of the derived values are given in Table II. The weighted mean values $\pm$ standard errors are: $V = 432.0 \pm 44.3$ mmol/cell unit per min and $K = 137.7 \pm 57.1$ mM, $V/K = 3.19 \pm 0.52$.

TABLE II

Results of equilibrium-exchange efflux experiments, given as mean $\pm$ S.E. B is the slope of the straight line relating the reciprocal slope of $\ln C_t$ vs t (which is equal to $(K+S)/V$) to S, obtained by regression analysis and is equal to $1/V$. A is the intercept of the same line and is equal to K/V and r is the correlation coefficient of the regression analysis.

Experiment No.	A (· 10)	B	r	V/K (min^{-1})	K (mM)	V (mmol/cell unit per min)
1	3.12 ± 1.04	0.119 ± 0.028	0.089	3.20 ± 1.06	157.1 ± 118.4	504.2 ± 118.6
2	2.96 ± 0.61	0.144 ± 0.017	0.096	3.38 ± 0.69	123.3 ± 70.1	416.7 ± 49.2
3	3.90 ± 1.91	0.125 ± 0.052	0.099	2.56 ± 1.25	187.2 ± 178.1	480.0 ± 199.7
Weighted Average				3.19 ± 0.52	137.7 ± 57.1	432.0 ± 44.3

Infinite-trans uptake

The use of this experimental procedure has not been reported hitherto for galactose but seemed desirable as an independent way to determine transport sites at the outer face of the membrane. One might expect that the uptake of labelled galactose should be linear with time over a certain time interval at all concentrations S_0. That this is so is demonstrated in Fig. 1. From such uptake curves was obtained the dependence of the rate of uptake on the extracellular concentration (Fig. 2). We subjected the data of Fig. 2 to a least square fit analysis of v against S_0 by means of the maximum neighbourhood method, using library program NONLSQ of the Hebrew University Computation Centre. Fitting to one specific site yielded the following values. $V = 262 \pm 4$ mmol/cell unit per min. $K = 25 \pm 1$ mM. When simple diffusion was added in parallel we got the following values: $V = 239 \pm 11$, $K = 21.5 \pm 2$ and the

passive permeability coefficient was 0.09 ± 0.04 min^{-1}. An F-test did show a significant improvement of fit upon the addition of the third parameter ($P < 0.05$). Addition of a fourth parameter, namely, trying to fit the results to a model of two specific sites in parallel, yielded results which were very uncertain (standard errors were larger than estimates and no improvement of fit could be obtained). This result is not surprising since the overall contribution of a low affinity site to the flux, under the maximal external galactose concentration tested, could not have been greater than the experimental error. Hence even powerful statistical analysis is unable to detect a second site.

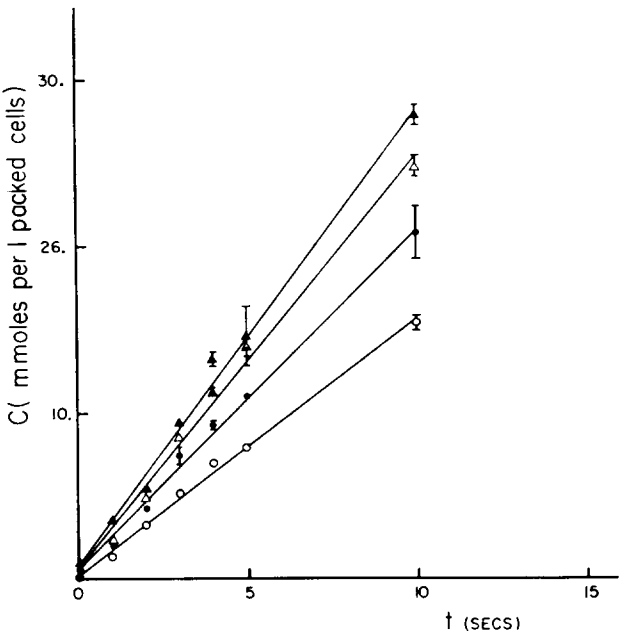

Fig. 1. Uptake of labelled galactose into cells preloaded with 250 mM non-labelled galactose as a function of time. Conditions: pH 7.4, 20 °C. Total osmolarity 560 mosM. Cells were washed and then equilibrated with 250 mM galactose. Cells were then centrifuged and the medium discarded. The highly concentrated suspension (hematocrit 80 %) was mixed vigorously with labelled galactose at different concentrations for various periods of time. Uptake was stopped and measured as described in methods. ○, 10.6 mM; ●, 14.5 mM; △, 22.3 mM; ▲, 37.1 mM. Values given are means ± S.E. of quadruplicates. Where S.E. is not shown it was smaller than the size of the symbol. Straight lines were obtained by regression analysis. All correlation coefficients were larger than 0.995.

DISCUSSION

The results of the present work demonstrate the following properties of the sugar transport system in human erythrocytes. Efflux of galactose is mediated by a low affinity site with a high maximal velocity. The V/K ratio of this site is 1.01 min^{-1}. The equilibrium exchange efflux results demonstrate a low affinity site although the K is lower than expected from the zero-trans efflux K. The maximal velocity obtained by this experimental procedure is almost twice that of the zero-trans efflux, which is as anticipated from the assumption that the rate limiting step now is the resistance to

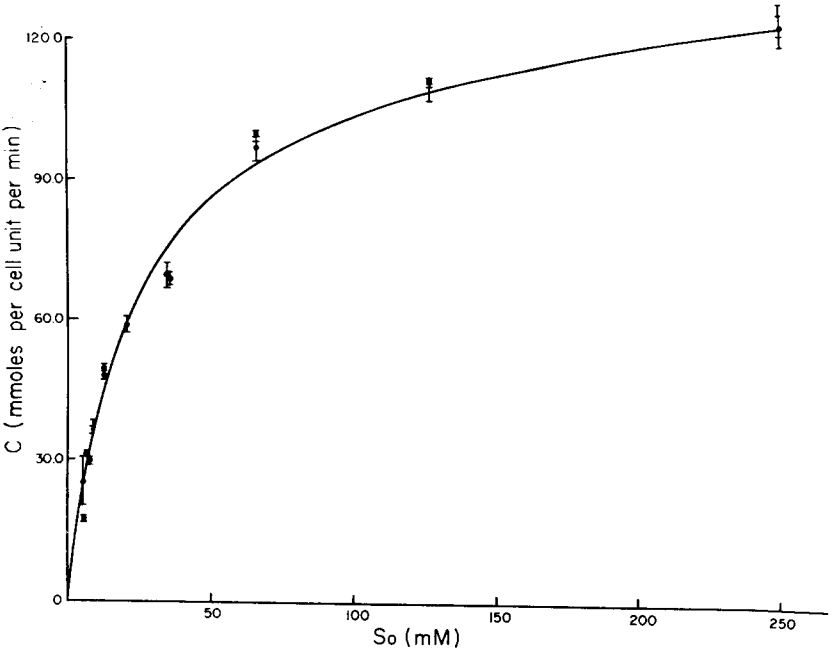

Fig. 2. Uptake of labelled galactose into cells preloaded with 250 mM galactose as a function of external galactose concentration. Conditions: pH 7.4, 20 °C, total osmolarity 560 mosM. Cells were washed and then loaded with galactose to 250 mM. Aliquots of concentrated suspension were mixed vigorously with labelled galactose at different concentrations for 5 s. Stopping and measurement of uptake are described in Methods. Results of two separate experiments on different batches of blood are given as mean ± S.E. of 4 experiments. The curve was calculated according to the equation $v = VS/(K+S)+aS$ and the parameters used were those described by regression analysis.

the migration of the loaded carrier which is lower than that of the unloaded one. The V/K ratio obtained by this procedure is very significantly greater than that of the zero-trans procedure, i.e. 3.19 min^{-1}.

The infinite-trans procedure yielded a maximal velocity very similar to that obtained in the zero-trans efflux procedure. The Michaelis constant derived by this procedure is similar to that obtained by Krupka [4] in an infinite-cis efflux experiment. Kinetic parameters of galactose transport system are collected in Table III.

In a previous publication [1] we reported a V/K ratio of 1.4 min^{-1} for zero-trans influx mediated by a high affinity site and a V/K of 0.075 min^{-1} for the influx on a low affinity site. Analysis of the conventional carrier [8] predicts that V/K ratios in both directions of transport should be equal for any carrier system. Thus from the V/K values (Table III) the high affinity site outside and the low affinity site inside are likely to be components of the same carrier system. Obviously such an asymmetry is reflected not only in the affinities but also in the maximal velocities so that efflux will be about 15 times faster than influx.

Taken together with the results of the previous paper [1] it is possible now to reject conclusively the model of a single asymmetrical carrier for sugar transport in human erythrocytes, the model treated (and rejected) by Hankin et al. [5]. This conclusion follows from the demonstration of high and low affinity sites at both the inner and the outer faces of the membrane.

TABLE III

Summary of kinetic parameters of the galactose transport system in human erythrocytes derived by different experimental procedures at 20 °C. Procedures used in the present work are marked (*). Other data come from a previous publication [1].

Procedure	V (mmol/cell unit per min)	K (mM)	V/K (min^{-1})	Site facing
Equilibrium-exchange efflux*	432	138	3.19	inside
Infinite-trans influx*	240	21		outside
Zero-trans efflux*	255	240	1.01	inside
Zero-trans influx	H = 16	H = 11	1.4	outside
	L = 21	L = 286	0.075	
Infinite-cis influx		21–25		inside

We must deal now with the possibility of two symmetrical or nearly symmetrical carriers in parallel. Were this model to hold, our infinite-trans and zero-trans uptake experiments would have revealed the substantial contribution of a low affinity site at the outer face of the membrane. Since this has not been found, this model must be rejected.

Another plausible model is that of two asymmetrical carriers in parallel but oppositely directed. Now for a single asymmetrical carrier it can readily be shown that the ratio V/K must be identical for the three procedures zero-trans influx and efflux and equilibrium exchange [5]. Our results show that V/K for equilibrium exchange is almost three times larger than V/K for zero-trans efflux. If the identity of V/K ratios were valid also for the two asymmetrical carriers model, we would be able to reject this model also. However, at present this remains an open question.

The tetramer model previously proposed from this laboratory [6] is in accord with the finding of high and low affinity sites on both sides of the membranes. However, the model must in its original form also be rejected. The model predicted an equality of zero-trans maximum velocities in both directions while from the present data and those of the previous report [1] the maximum zero-trans efflux is some 7–8 times larger than that of the influx. In addition, the prediction that the low affinity site should make the major contribution to the uptake in the infinite-trans conditions [7] is not confirmed by the present results. Possibly some modification of the original tetramer hypothesis might yet explain all the findings.

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