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Galactose uptake by human platelets in vitro

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Galactose transport by human platelets has been studied by measuring the cellular accumulation of the radiolabeled sugar during brief periods of suspension in varying concentrations of galactose. Weighted least-squares regression curves fitted to the measurements (initial velocity versus galactose concentration) indicate that a kinetic model with two saturable components is statistically more consistent with the data than a model based upon a single process ($P < 0.001$). For the two-component model $K_{m1} = 0.29$ mM, $V_1 = 1.2$ mmol/min per 10^{15} platelets, $K_{m2} = 46$ mM, $V_2 = 117$ mmol/min per 10^{15} platelets. The fact that galactose metabolites did not accumulate during the initial phase of uptake indicates that the uptake process is not mediated by enzymatic catalysis. Surface binding also appears inadequate to explain the uptake. The most likely basis for the kinetic data, therefore, is membrane transport. The kinetics are consistent with transport by coexistent membrane structures as well as with transport by a single structure manifesting negative cooperativity.

Introduction

Although platelets are known to metabolize galactose and incorporate it into glycogen [1], the mechanism by which these cells initially absorb galactose from their environment, i.e., galactose transport, has not been specifically studied. It has been shown, however, that accumulation of galactose and other hexoses in platelets can be inhibited by the glycoside phlorizin [2]. Therefore, the uptake process cannot be explained by simple diffusion alone. Furthermore, glucose, mannose and fructose, but not galactose, compete with 2-deoxyglucose for entrance into platelets [3]. Galactose, therefore, is apparently excluded from an uptake pathway shared by the other hexoses.

Much more information is available about sugar uptake by erythrocytes, which transport galactose from their medium by two kinetically distinct and saturable mechanisms [4]. Because of the ontogenetic kinship of red cells and platelets, we reasoned that platelets might have a similar transport system for galactose. In the report that follows, we present evidence that this is indeed the case [5].

Materials and Methods

Materials. D-[1-^{3}H]Galactose (10.4 Ci/mmol), D-[1-^{14}C]galactose (58 Ci/mol), L-[1-^{14}C]glucose (58 Ci/mol), and NCS Tissue Solubilizer were purchased from Amersham (Arlington Heights, IL). Econofluor, Aquasol and 5-[1,2(n)-^{3}H]serotonin (20 Ci/mmol) were from New England Nuclear Corp. (Boston, MA). D-Galactose, D-glucose, L-glucose, D-galactose 1-phosphate, and

Abbreviations: [^{3}H]galactose, D-[1-^{3}H]galactose; [^{14}C]galactose, D-[1-^{14}C]galactose; L-[^{14}C]glucose, L-[1-^{14}C]glucose; [^{3}H]serotonin, 5-[1,2(n)-^{3}H]serotonin. Unless otherwise specified all sugars are of the D-configuration.

phlorizin were products of Sigma Chemical Corp. (St. Louis, MO). Bovine serum albumin, fraction V, was obtained from Miles Laboratories (Kankakee, IL). Pyridine and ethyl acetate were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Polycarbonate filters, 0.4 μm pore-size, were manufactured by the Nucleopore Corp. (Pleasanton, CA). Chromatographic sheets precoated with 0.1 mm cellulose were obtained from E. Merck (Darmstadt, F.R.G.).

Platelet preparation. Human platelets were separated from fresh blood as previously described [6] and suspended at room temperature in glucose-free Tyrode's buffer [7] containing 5% bovine serum albumin or in phosphate-buffered saline with 10 mM EDTA (120 mM NaCl/10 mM sodium phosphate/10 mM Na_2EDTA (pH 7.4)). Measured with a Celloscope (Particle Data, Inc., Elmhurst, IL) the final platelet concentration was 10^9/ml. Contaminating red or white cells were fewer than one per 10^6 platelets.

Transport rate experiments. A stock solution of 270 mM [^{3}H]galactose (0.55 Ci/mol), 30 mM Tris (pH 7.4) was stored at −20°C. This was diluted with phosphate-buffered saline (140 mM NaCl/10 mM sodium phosphate (pH 7.4)) to give galactose concentrations of 0.02–200 mM, which were confirmed by measuring the isotope dilution. Trace amounts of [^{3}H]galactose were added to the highest dilutions ($\leq$ 10 mM galactose) to produce specific activities of 0.01–1 Ci/mmol. Identical dilutions were made with phosphate-buffered saline with 10 mM EDTA (pH 7.4).

To measure the rate of uptake of galactose by platelets, 25 μl of a [^{3}H]galactose solution was mixed by rapid pipetting with an equal volume of platelet suspension at room temperature for intervals up to 60 s. Uptake was stopped by diluting the platelets 200-fold with 10 ml of ice-cold Tris-buffered saline (100 mM NaCl/40 mM Tris-HCl/10 mM Tris base (pH 7.4)) quickly delivered with a Repipet (Lab Industries, Berkeley, CA). The diluted suspension was aspirated through a 0.4 μm-pore polycarbonate filter. The time between addition of the cold diluent and completion of filtration was always less than 30 s. The 0.4 μm-pore filters were shown to retain 100% of the platelets.

Each filter was dissolved in 0.5 ml of NCS Tissue Solubilizer by incubation at 50°C for approx. 10 min. The alkaline NCS was then neutralized with 25 μl of glacial acetic acid and mixed with 5 ml of Econofluor for scintillation counting with a Mark III Liquid Scintillation System (Tracor Analytic, Inc., Elk Grove Village, IL). Counting efficiency was measured with an external standard, and ^{3}H activity expressed as disintegration per minute (dpm).

To measure extracellular galactose trapped on the filters (equivalent to platelet uptake of galactose at time 0), 25 μl of the [^{3}H]galactose solutions was diluted in 10 ml of ice-cold Tris-buffered saline before the addition of 25 μl of the platelet suspension. The diluted suspension was then quickly filtered and processed as outlined above.

Least-squares regression lines were fitted to plots of filtered disintegrations per minute versus time over the initial 10 s of platelet exposure to [^{3}H]galactose. From the slopes of these lines initial velocities (v) of galactose uptake were calculated and expressed as mmol/min per 10^{15} cells.

To study the rate of galactose efflux from the platelets during dilution in cold buffer, 50 μl samples of [^{3}H]galactose-platelet suspension (pre-incubated for 15 s at room temperature) were diluted in 10 ml of ice-cold Tris-buffered saline for intervals up to 3 min before filtration.

Because of the possibility of platelet rupture during filtration with consequent loss of intracellular galactose, the filtration method was compared with separation of platelets by centrifugation in ice-cold buffer. Platelets which had been incubated in [^{3}H]galactose for intervals up to 2 h and separated from their suspending medium by either of the methods were shown to contain equivalent amounts of radiolabeled sugar, although background (extracellular) radioactivity was higher with the centrifugation technique. Platelets were also labeled by incubation with [^{3}H]serotonin (2 ml platelet-rich plasma, 1 μCi [^{3}H]serotonin, 37°C for 1 h), washed, and separated from their buffer both by filtration and centrifugation, again with equivalent results. Therefore, there was no evidence that intracellular [^{3}H]galactose was lost by cell rupture during trapping on the polycarbonate filters.

Measurements of diffusional transport. The contribution of simple diffusion to total galactose

transport was quantitated by incubating 25 μl of platelet suspension with an equal volume of 2–200 mM L-[^{14}C]glucose for intervals up to 10 min before dilution with 10 ml of cold Tris-buffered saline and filtration. Initial uptake rates were calculated as described above.

Mathematical analysis of uptake. With the LIGAND computer program [8] weighted least-squares regression lines were fitted to the isotherm of initial uptake velocity, v, versus extracellular galactose concentration, S, by adjusting the parameters K_m and V, according to the formula

$$v = \frac{VS}{K_m + S}$$

where V is the maximal initial velocity of uptake, and K_m the galactose concentration (S) at which initial uptake velocity is half maximal. With the F-test the fit of a single rectangular hyperbola (Model 1) was compared statistically with the fit of a curve representing the sum of two rectangular hyperbolas (Model 2) [8].

Pre-loading platelets with galactose. A portion of a routinely prepared platelet suspension was incubated for 30 min at 37°C with 10 mM unlabeled galactose. Control platelets were incubated without the added sugar. After cooling to room temperature, aliquots of both samples were mixed with [^{3}H]galactose solutions to give a final galactose concentration of 5 mM in each. Initial transport rates were measured as described above.

Thin-layer chromatography. Platelets suspended (10^9/ml) in 50 μl of Tyrode's buffer with 5% bovine serum albumin or in 50 μl of phosphate-buffered saline with 10 mM EDTA were mixed with 50 μl of phosphate-buffered saline (with or without 10 mM EDTA) containing 10 μCi of [^{14}C]galactose (58 Ci/mol) for intervals of 10 s at room temperature. Each suspension was then rapidly mixed with 10 ml of ice-cold tris-buffered saline which contained 10 μCi of [^{3}H]galactose to serve as a marker of extracellular sugar. The diluted suspensions were filtered by suction within 30 s through 0.4 μm-pore polycarbonate filters, which were quickly transferred to 1 ml volumes of 10% trichloroacetic acid and rocked at 4°C for 30 min. After the filters had been removed, the acid was extracted from the eluate with ether. The eluates were evaporated to dryness and redissolved in 0.1 ml volumes of water. Any particulate matter was removed by centrifugation. Authentic galactose was added as an internal standard before the samples were spotted on thin-layer cellulose. A sample of galactose 1-phosphate was spotted in a separate lane.

Chromatographic separation was performed with a solvent composed of pyridine/ethyl acetate/water (5 : 18 : 6, v/v/v). After three passes of the solvent, the lanes containing the unknowns were stained for neutral sugar by dipping in a solution of 35 mM $AgNO_3$ in 1% water/99% acetone, followed by drying and dipping in 0.5 M NaOH in 95% ethanol. Staining was terminated by washing with 0.3 M $Na_2S_2O_3$. The galactose 1-phosphate standard was localized by straying with the Hanes molybdate reagent [9].

After staining, each lane was cut into 0.5 cm pieces which were rocked in 1 ml volumes of water at room temperature for 2 h. 10 ml of Aquasol was then added to each sample, which was assayed for ^{3}H and ^{14}C by liquid scintillation counting.

A portion of the ^{14}C in each chromatographic lane represented extracellular [^{14}C]galactose retained with the filtered platelets. This contaminating [^{14}C]galactose was identified by its co-migration with residual [^{3}H]galactose which had been introduced with the cold diluting buffer. From the ratio of ^{3}H to ^{14}C in the diluted platelet suspension (approximately 1 : 1) and the concentration of ^{3}H in the slices of the chromatographic lanes, the contribution of contaminating extracellular [^{14}C]galactose to the total ^{14}C in each slice could be calculated. This was subtracted from the total ^{14}C to give the amount of cell-derived ^{14}C in each slice.

Results

[^{3}H]Galactose uptake by platelets

Accumulation of [^{3}H]galactose by platelets was measured for 60 s but was found to remain linear only over the initial 15–20 s at high and low concentrations of galactose (Fig. 1). All subsequent rate measurements, therefore, were based upon uptake during the first 10–15 s of galactose exposure to the platelets.

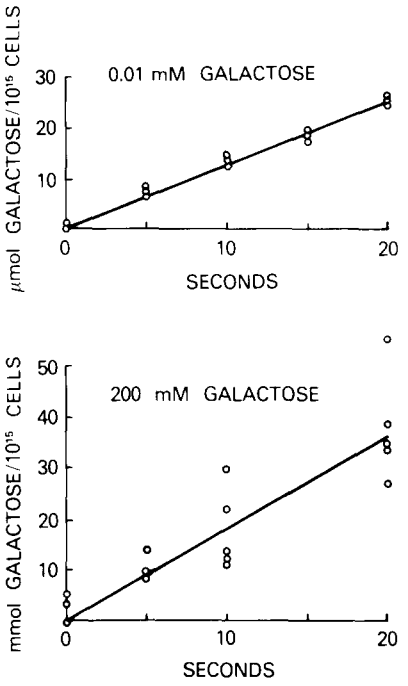


Fig. 1. Accumulation of galactose by platelets over 20 s in a solution of 0.01 mM or 200 mM galactose at room temperature. The data have been fitted with least-squares regression lines.

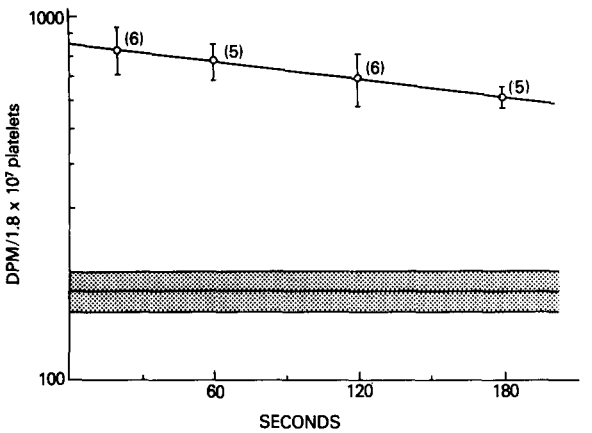


Fig. 2. Efflux of [^{3}H]galactose from platelets suspended in ice-cold buffer. Platelets were pre-incubated for 15 s at room temperature with 0.1 mM [^{3}H]galactose and then diluted with 10 ml ice-cold buffer for the time intervals shown (abscissa) before filtration. ^{3}H remaining with the platelets is shown on the ordinate as disintegration per min (dpm)/$1.8 \cdot 10^7$ platelets and expressed as the mean ± S.E. based upon the number of replicates shown in parentheses. Background disintegrations per min (residual extracellular ^{3}H) is shown as the shaded area, representing a mean ± S.E. based on four replicates.

Loss of platelet [^{3}H]galactose during dilution

Platelets exposed to 0.1 mM [^{3}H]galactose or 100 mM [^{3}H]galactose for 15 s and then suspended in ice-cold buffer showed a gradual exponential loss of ^{3}H during an interval of dilution as long as 3 min. The results with 0.1 mM [^{3}H]galactose are shown in Fig. 2. The earliest measurements of platelet [^{3}H]galactose were obtained following a dilution time of 20 s. Extrapolation from these data indicates that with extracellular galactose concentrations of 0.1 mM or 100 mM approximately 6% of the original cellular [^{3}H]galactose was lost during the initial 30-s dilution in cold buffer. Since in measurements of uptake rate the dilution periods were never over 30 s, the effect of efflux on the rate measurements was ignored.

Uptake of L-[^{14}C]glucose by platelets

The accumulation of L-[^{14}C]glucose by platelets remained linear for as long as 6–10 min. Based on 13 studies the rate constant of initial L-glucose

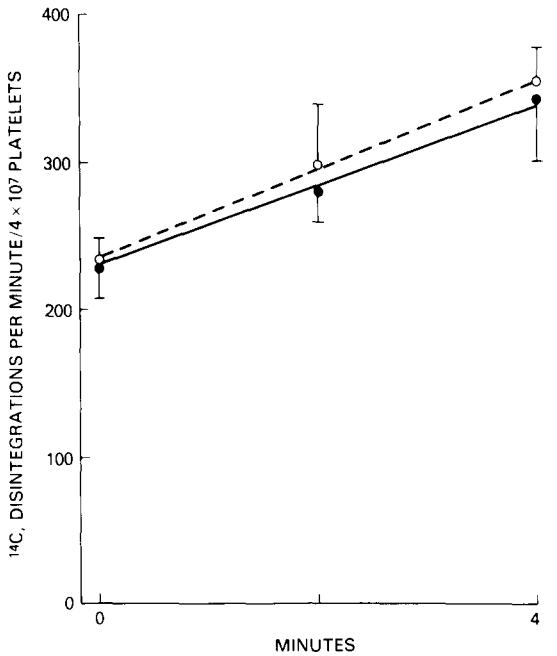


Fig. 3. Accumulation of L-[^{14}C]glucose by platelets in a solution of 1 mM L-glucose (○— — —○) and 100 mM L-glucose (●———●). The concentration of L-[^{14}C]glucose was the same in both solutions. Points are shown as the means of four replicates ± S.E. The data have been fitted with least-squares regression lines.

uptake was 0.12 ± 0.083 (S.E.) l/min per 10^{15} platelets. It was unaffected by L-glucose concentration over a range of 1 to 100 mM (Fig. 3). This observation indicates that L-glucose uptake occurs by passive diffusion.

Dependence of initial velocity (v) on galactose concentration (S)

Results obtained with platelets suspended in phosphate-buffered saline with 10 mM EDTA ($N = 50$ studies) were indistinguishable from the results with platelets suspended in the more physiologic Tyrode's buffer with albumin ($N = 24$) (Fig. 4). Therefore, both sets of data were pooled for mathematical analysis. A graph of v versus S appeared hyperbolic (Fig. 4). Transforming the data as v/S versus v produced a biphasic plot (Fig. 5) [10].

A mathematical model based upon two saturable components (Model 2) fit the data significantly better than a model based upon one component (Model 1): $F = 18.2$, $P < 0.001$. A component of unsaturable (diffusional) uptake measured experimentally with L-glucose was included in both models (Table I).

The maximal initial velocity of uptake (V) and

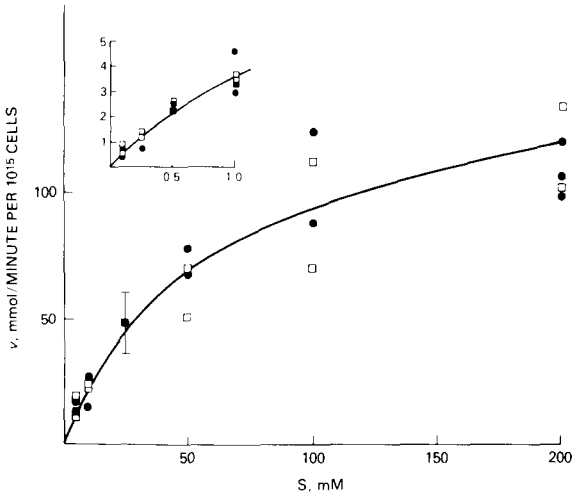


Fig. 4. Initial velocity of uptake, v, versus extracellular galactose concentration, S. The data have been fitted with a curve based upon a two-transporter model (Model 2, Table I). ●, points from studies performed in Tyrode's buffer with albumin. □, points obtained with platelets in phosphate-buffered saline and EDTA. Measurements made with 25 mM galactose ($N = 17$) are represented as the mean ± S.E. This group includes studies performed in both buffer systems. The inset displays the data for the lower galactose concentrations. Studies with 0.01 mM galactose ($N = 18$) have been omitted.

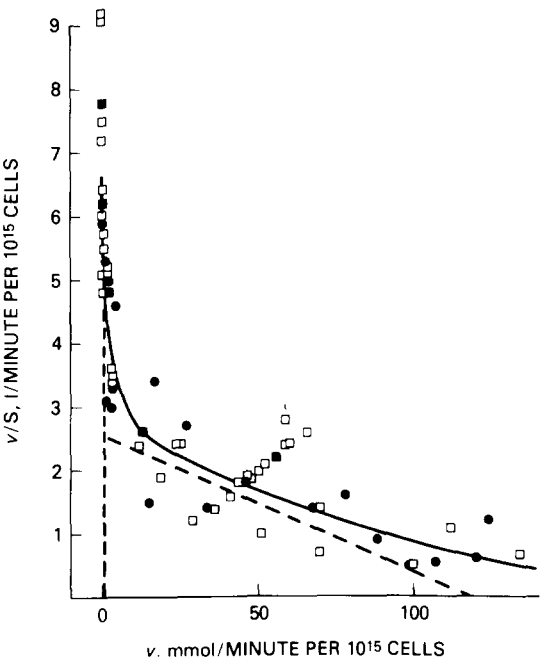


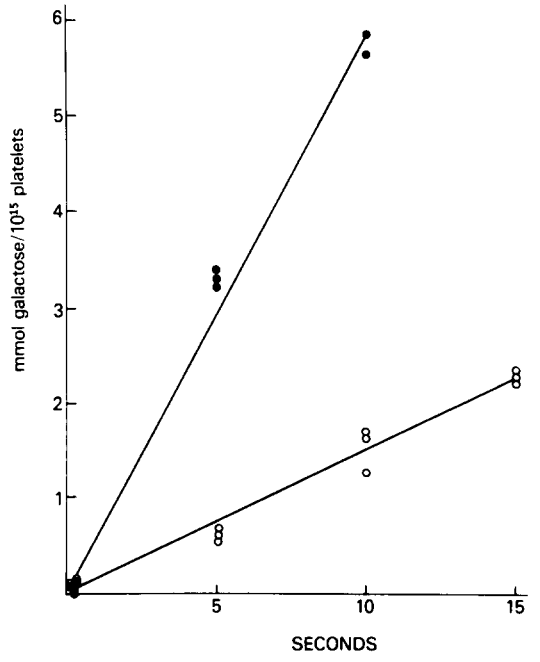
Fig. 5. v/S versus v. The data have been fitted with a curve based upon a model of two saturable components plus an element of diffusional uptake (Model 2, Table I). ●, points from studies performed in Tyrode's buffer. □, points obtained with platelets in phosphate-buffered saline and EDTA. The individual saturable components are shown as dashed lines.

TABLE I

KINETIC MODELS OF GALACTOSE UPTAKE

These models are based upon weighted least-squares regression curves fitted with the computer program LIGAND [8]. Model 2 gives a significantly better fit ($F = 18$, $P < 0.001$). The diffusion constant 0.12 l/minute per 10^{15} platelets was determined experimentally with L-glucose. K_m is expressed as mM, and V as mmol/min per 10^{15} platelets. Coefficients of variation for the fitted parameters are shown in parentheses.

Model		
(1) $v = \frac{VS}{K_m + S} + 0.12S$	$V = 98$ (6%)	$K_m = 25$ (11%)
(2) $v = \frac{V_1 S}{K_{m1} + S} + \frac{V_2 S}{K_{m2} + S} + 0.12S$	$V_1 = 1.2$ (61%)	$K_{m1} = 0.29$ (71%)
	$V_2 = 117$ (7%)	$K_{m2} = 46$ (15%)



the galactose concentration at which the velocity is half maximal (K_m) were calculated for the single-component model and for the high-affinity (V_1; K_{m1}) and low-affinity (V_2, K_{m2}) components of the more complex model (Table I). Although the latter model fits the data significantly better, its greater complexity leads to a larger uncertainty in the estimates of the transport parameters, as shown by the relatively high coefficients of variation for V_1 and K_{m1} (Table I) [8].

In Figs. 4 and 5 the data have been fitted with the curve for the summation of two saturable components plus the diffusional component (Model 2, Table I). In Fig. 5, the individual saturable components of that system are also

Fig. 6. Effect of pre-incubation with unlabeled galactose. Platelets were incubated at 37°C for 30 min in Tyrode's buffer with 5% bovine serum albumin with (●) or without (○) 10 mM galactose and then diluted at room temperature with [^{3}H]galactose to give a final galactose concentration of 5 mM.

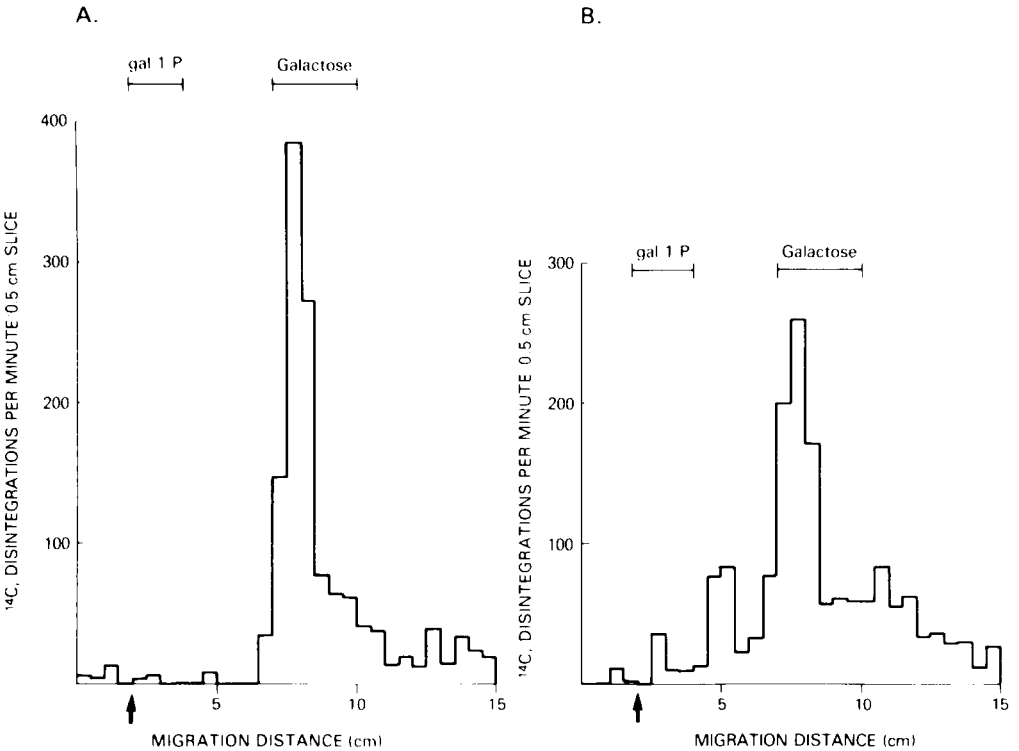


Fig. 7. Thin-layer chromatography of platelet extracts obtained after the cells were exposed to [^{14}C]galactose for 10 s. (A) Platelets suspended in phosphate-buffered saline with 10 mM EDTA. (B) Platelets suspended in Tyrode's buffer with albumin. Residual extracellular [^{14}C]galactose has been subtracted (see text). The origins are indicated by arrows.

shown. The horizontal intercepts of the straight lines give V_1 and V_2, while their slopes are $-K_{m1}^{-1}$, and $-K_{m2}^{-1}$ [10].

Effect of pre-loading platelets with galactose

Preincubation of platelets in 10 mM unlabeled galactose produced an approximately 4-fold increase in uptake of 5 mM [^{3}H]galactose as compared with uptake by control platelets (Fig. 6).

Thin-layer chromatography

In buffer with and without EDTA, the predominant radiolabeled constituent from platelets incubated for 10 s with [^{14}C]galactose co-migrated with authentic galactose (Fig. 7). In the solvent system employed, galactose-1-phosphate, the earliest metabolite of galactose, does not migrate. Although minor amounts of ^{14}C remained at the origin of the sample prepared in Tyrode's buffer, virtually no radioactivity remained at the origin of the sample prepared in EDTA. Therefore, there was little evidence of galactose metabolism occurring during the 10-s incubation periods at room temperature, particularly in the presence of EDTA.

Discussion

The experiments reported here provide the basis for a kinetic model of galactose uptake by human platelets under 'zero-trans' conditions, that is, when initial intracellular galactose concentration (S_i) is assumed to be zero and to remain negligible during the experimental interval, while the specified extracellular concentration of galactose (S) is essentially constant. The data consist of rate measurements taken during the first 10 s of exposure to the sugar, when platelet galactose was observed to increase linearly (Fig. 1). With this experimental design the initial uptake velocity, v, approached a maximum as extracellular galactose concentration was increased (Fig. 4), thereby excluding simple diffusion as an adequate explanation for the observations. Nevertheless, it is reasonable to assume that diffusion accounts for a minor component of uptake equivalent to that measured experimentally with L-glucose. To account for the remainder of the uptake, we propose that the extracellular sugar (S) rapidly interacts with structures (T) exposed at the platelet surface, leading to steady-state concentrations of ST, and that the linear accumulation of galactose over the 10-s period of measurement is directly proportional to the degree to which the structures involved are saturated:

$$S + T \rightleftharpoons ST \rightarrow S_i + T \quad (1)$$

The accuracy of the experimental method depends upon the ability to arrest cellular uptake abruptly at specific times and to prevent loss of cellular galactose before the platelets are isolated from their suspending medium. Although the sudden 200-fold dilution of the platelet suspension with ice-cold buffer should reduce subsequent uptake to negligible levels, no such assumption can be made about efflux from the cells since the dilution suddenly changes the transmembrane gradient of galactose to favor net flow outward. However, loss of [^{3}H]galactose from pre-loaded platelets suspended in ice-cold buffer for 30 s was measured to be on the order of 6% of the intracellular level at the moment of dilution (Fig. 2). This loss was considered to be sufficiently small to be ignored in the rate calculations. It would appear, therefore, that the method employed fulfills the requirements for accurate rate measurements.

The saturation kinetics demonstrated by galactose uptake under the experimental conditions of this study are consistent with several physical processes, including enzyme catalysis, ligand binding, and transport. Several lines of evidence, however, favor transport (i.e., movement of the unaltered sugar through the plasma membrane) as the most probable. The possibility that enzyme catalysis underlies initial uptake appears remote because of the failure to demonstrate significant galactose metabolites during the initial uptake interval. Thin-layer chromatography of extracts from platelets incubated with galactose under conditions identical to those for the rate measurements revealed that the free sugar was the dominant radiolabeled constituent, especially in the presence of EDTA (Fig. 7). Although the chromatographic analysis was performed with galactose of higher specific activity and lower concentration than any used to measure the uptake rates, the results are valid regardless of S, if we make the reasonable assumption that [^{14}C]galactose competes equally

with the unlabeled sugar. Additional evidence against enzyme catalysis is the observation that v is increased when the platelets have been preloaded with galactose and then diluted to produce a transient gradient, decreasing from inside to outside the cell (Fig. 6). Under such conditions enzyme-mediated uptake of the radiolabeled sugar might be inhibited by competition with the unlabeled sugar, but it is difficult to envision how enzyme-mediated uptake might be accelerated.

Simple binding of galactose to saturable sites on the platelet exterior also appears to be an unlikely explanation for the kinetics because of the acceleration of uptake observed in the setting of a decreasing inside-outside gradient of unlabeled sugar (Fig. 6). Furthermore, preliminary studies in our laboratory have shown that v is unaffected by extensive proteolysis of the platelet surface (unpublished data). Therefore, any binding of galactose to the plasma membrane presumably must occur at sites inaccessible to extracellular protease. Such sites, sequestered in the lipid bilayer, would seem unlikely, unless binding there were the first step in the movement of the sugar through the membrane.

We feel, therefore, that the uptake kinetics we have observed most reasonably reflect platelet membrane transport of galactose; that is, that the velocity measurements, v, correspond to movement of galactose through the plasma membrane of the cells with subsequent release of the sugar into the cytosol, according to the simplified scheme (1) given above. Such a scheme is compatible with a model of the transporter (T) as a simple pore [11]. However, the observation that v is accelerated by the presence of intracellular galactose (Fig. 6) argues against a simple-pore model and favors a more complex system, namely the carrier model of transport, which hypothesizes an intramembranous structure shuttling sugar molecules between the outer, T_0, and inner, T_i, surfaces of the plasma membrane [11,12]:

$$\begin{array}{ccc} T_0 & \rightleftharpoons & T_i \\ \pm S \downarrow\uparrow & & \uparrow -S_i \\ [ST]_o & \rightleftharpoons & [ST]_i \end{array} \qquad (2)$$

Acceleration of influx by the presence of a decreasing inside-to-outside gradient of galactose (Fig. 6) implies that when associated with the sugar, the transporter moves through the membrane more rapidly than when it is free of sugar. Therefore, a net efflux of sugar leads to a relative increase in free carrier concentration at the outside surface (T_o), thereby promoting influx of S. (For a more complete discussion of this phenomenon, the reader should consult Ref. 12.) It is of interest that the only other cell in which intracellular sugar has been demonstrated to accelerate hexose uptake is the human red cell [11,13,14], which of course is ontogenetically closely related to the human platelet studied here.

Another similarity between red cells and platelets is that both appear to transport galactose by a system with multiple kinetic component; i.e., the reaction schemes shown above are somehow duplicated. This was demonstrated earlier for human red cells by Ginsburg and Stein [4]. Heterogeneous mediation of transport is also well established for amino acids in many tissues [15]. The heterogeneity of galactose transport by platelets is implied in our study by the fact that the mathematical relationship of v to S is described significantly better by the sum of two rectangular hyperbolas than by a single hyperbola ($P < 0.001$). This is most obvious in a plot of v/S versus v (Fig. 5).

The physical interpretation of this dual system must for the moment remain ambiguous. However, the kinetic data are consistent with coexistent membrane structures for transporting galactose, each with a distinct affinity and capacity for the sugar. On the other hand, the data are also compatible with a single transporting structure with an affinity and capacity for galactose which depend on the extracellular galactose concentration (negative cooperativity). The elucidation of the true mechanism will probably require isolation of the structures involved followed by functional studies of the components in simplified systems.

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