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Failure of Equilibrium Dialysis to Show Selective Monosaccharide Binding by Erythrocyte Membranes

Stanley J. Masiak and Paul G. LeFevre

Department of Physiology and Biophysics, Health Sciences Center, State University of New York, Stony Brook, New York 11790

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Summary. Equilibrium dialysis has been reported to show stereoselective binding of the preferred sugar transport substrate, D-glucose, by NaI protein extracts of human erythrocyte membranes. However, we were unable to show any detectable differential binding of D-glucose (as compared with the poorly transported analogue, L-sorbose) with NaI protein extracts. The basis for this decided dissonance is not clear. Extracts with nonionic detergents, various alcohols, and pyridine were also used, but the results with these were also negative. Our data indicate either that the transport sites are not thus extractable in a functional condition, or that only a very small number of binding sites (less than 100,000) are involved with the sugar translocation; and that this method cannot serve to measure the site population unless a far greater concentration of the binding material can be achieved than has so far been possible.

Over the past several years, attempts have been made to demonstrate the binding of sugars to erythrocyte membranes as a basis for the identification of a stereoselective membrane component involved in the translocation of simple sugars between the interior and exterior of human erythrocytes (Bobinski & Stein, 1966; Bonsall & Hunt, 1966; Levine & Stein, 1967; LeFevre & Masiak, 1970). However, the initial method of retardation chromatography was inadequate because the ghost preparations were still sufficiently structured that a functional transport system could still operate to convey the sugars into and out of closed compartments (membrane vesicles) on the columns (LeFevre & Masiak, 1970). Because of the possibility of this type of interpretation of their results, Levine and Stein (1967) presented direct observations of selective binding of D-glucose (as compared with the poorly transported analogues, L-sorbose or L-glucose) by ghost protein extracts, by means of ultrafiltration and equilibrium dialysis techniques.

The present study was undertaken in the hope of confirming and perhaps extending these observations through the use of selected protein extraction procedures which have been shown, in some instances, to be sufficiently gentle to allow continued enzyme activity in the resulting extract. Unfortunately, we have been unable to demonstrate *any* selective binding of D-glucose, irrespective of the extraction procedure or of the amount of concentrated extract used. This lack of preferential binding appeared under circumstances where a 1% difference would correspond to only 25,000 binding sites per cell. While it is difficult to specify precisely the limits of uncertainty in our dual-channel counting, it is unlikely that we could have failed to resolve a D-glucose retention on the order of 100,000 sites per cell.

Materials and Methods

Human erythrocytes were taken from out-dated blood in standard acid-citrate-dextrose bags obtained from either the American Red Cross or the Nassau-Suffolk (N.Y.) Inter-County Blood Bank. When NaI extracts were to be used, the ghost preparation and extracts followed the methods given by Levine and Stein (1967); these extracts were dialyzed for 36 to 48 hr against distilled water and lyophilized. The dried material was resuspended in 2.5 or 3 mM sodium phosphate buffer at pH 7.0. For extracts with Triton X-100 (Rohm and Haas Co.) procedures for both ghost preparation and extraction were taken from Bonsall and Hunt (1966). For extracts with a variety of alcohols, procedures for each ghost preparation and extraction were taken from Maddy (1964), Morgan and Hanahan (1966), Rega, Weed, Reed, Berg and Rothstein (1967), and Zwaal and Van Deenen (1968). For extractions with pyridine, procedures for both ghost preparation and extraction were taken from Blumenfeld (1968). For extracts with Lubrol WX, procedures for both ghost preparation and extraction were taken from Medzihradsky, Kline and Hokin (1967). Several additional alcohols, Lubrol MOA, Tween 20, and Tween 80 (Mann Research Lab.) were also used for ghost extraction. Erythrocyte ghosts were prepared according to the method of Masiak and Green (1968) and the extraction procedures were comparable to the appropriate alcohol or detergent extraction procedures cited above.

The alcohol and detergent extracts were usually concentrated to 1.1 ml by ultrafiltration (Diaflo Cell, Amicon Corp.). The concentrated protein extracts (1 ml) were placed in one side of an equilibrium dialysis cell (Chemical Rubber Co.) and in the other half of the cell was placed 1 ml of a mixture of ^{3}H-D-glucose (Nuclear-Chicago, or Amersham/Searle Corp.) and ^{14}C-L-sorbose (Nuclear-Chicago, or Calbiochem), each at 0.55 mM. The cell was allowed to rotate for a minimum of 17 hr at 4 °C. The mixtures from each side of the cell were removed with a syringe and the distribution of the sugars was determined by dual-channel counting in either a Nuclear-Chicago or a Picker Nuclear (Liquimat 330) liquid scintillation spectrometer. The scintillation mixture was 4 g of 2,5-diphenyl-oxazole plus 60 g of naphthalene per liter of 9:1 dioxane/methanol. Data were analyzed by suitable programming of the DAC-512 computer attachment of the Picker Liquimat 330 instrument.

The radiochemical purity of the ^{3}H-glucose and the ^{14}C-sorbose was assayed chromatographically on Whatmann 3 MM paper, with *n*-butanol/ethanol/water (52:33:15) as developer. The sugars were detected by the $AgNO_3$ method (Partridge, 1966), and small

sections of the chromatograms were counted to ascertain the localization of the radioactivity in the region of the sugar spot. Localization was quite satisfactory.

The protein determinations on all extracts and ghost preparations were by the method of Lowry, Rosenbrough, Friar and Randall (1951).

Results

Our findings with overnight dialysis on concentrates from NaI extracts, prepared exactly as recommended by Levine and Stein (1967), are summarized in Table 1. The net differences in the distribution of the two labeled sugars are expressed as percentages of the relative "glucose excess" on the side of the chamber containing the NaI extract (which usually contained 30 to 50% of the original crude ghost protein). The results *fail to demonstrate* any specific retention. We have not been able to account for the apparent experimental conflict with Levine and Stein (1967).

Also, white ghosts were extracted with several nonionic detergents which have been used to solubilize various enzyme systems from membranes. Our results, presented in Table 2, again fail to show any specific retention. Solubilization of the membrane protein is generally satisfactory with these

Table 1. Equilibrium dialysis with NaI extracts of Levine-Stein (crude) ghosts

ml Cells per ml extract	$[G]=[S]$ (mM)	Rel. G excess in extract (%)
3.3	0.21	1.8
5.0	0.22	−0.8
5.0	0.22	1.2
17.3	0.23	1.1
23.0	0.4	−2.8
23.0	0.4	2.7

Table 2. Equilibrium dialysis with detergent[a] extracts from white ghosts

Agent	% G excess	Agent	% G excess
Triton X-100	−0.4	Lubrol WX	−2.0
Triton X-100	−1.4	Lubrol WX	−2.0
Tween 20	0.1	Lubrol WX	1.9
Tween 80	0.1	Lubrol WX	−0.8
Lubrol MOA	1.4	Lubrol WX	1.2

Final concentration of all detergents was 1%. Final concentration of all sugars was 0.23 mM.

Table 3. Equilibrium dialysis with alcoholic extracts from white ghosts

Alcohol	% G excess	Alcohol 1:1 mixtures	% G excess
n-Butyl	−0.8	*n*-Butyl/*n*-Amyl	2.9
n-Butyl	0.5	*n*-Butyl/*n*-Amyl	−2.2
n-Butyl	0.2	*n*-Butyl/*n*-Amyl	−3.0
n-Amyl	1.4	*n*-Butyl/iso-Propyl	0.0
n-Amyl	−0.3	*n*-Butyl/*n*-Propyl	−0.6
n-Amyl	−1.9		
iso-Amyl	−0.7		

detergents, especially the lubrol series in which 90% of the original membrane protein remained in the supernatant after 1 hr centrifugation at $40{,}000 \times g$.

A variety of alcohols have also been used to extract proteins from membranes and Table 3 summarizes our findings with the most commonly used agents of this type. Again, no definitive "glucose excess" was evident. Most of the alcohols gave a 60 to 70% protein recovery in the extracts. *n*-Amyl alcohol, however, solubilized 90% of the original protein when the ghost to alcohol ratio was 1:1 (v/v). When the alcohol mixtures were used, a substantially lower amount of protein (35 to 45%) was solubilized. On several occasions the material at the interface between the alcohol and water layers was dried and resuspended in a small volume of water to test for preferential glucose binding. The results from these experiments were also negative.

Pyridine extraction by the method of Blumenfeld (1968) was equally unrewarding for demonstrating a "glucose excess" in the extracted material.

Discussion

The protein extracts from erythrocyte ghost preparations gave no evidence whatsoever of preferential binding of D-glucose in comparison to the relatively nontransportable analogue, L-sorbose. This seems quite surprising when one calculates the number of binding sites compatible with the lack of detectability, if all sites had actually been extracted with their binding properties intact. The number of binding sites per cell per 1% "glucose excess" observed can be obtained from the following relationship:

$$\frac{6 \times 10^{15} \times \underset{(\text{ml})}{\text{Vol}} \times \underset{(\text{mM})}{(K + [G])}}{\text{No. of Cells Represented}} \qquad (1)$$

Table 4. Apparent direct conflict in binding studies with NaI extracts of crude ghosts

ml Cells per ml extract	[G] (mM)	Vol (ml)	G-excess (%)	Sites/cell $\frac{}{(K+[G])}$
		Levine and Stein-Ultrafiltration		
3–5	0.20–0.28	0.8–1.0	7.0, 8.8, 15.3	$1.2–1.8 \times 10^6$
19	1.0–4.0	0.8	7.1, 9.5	$1.8–2.4 \times 10^5$
Best estimate sites/cell ca. 1.5×10^6				
		Masiak and LeFevre-Equilibrium dialysis		
3.3–5	0.21–0.23	1.0	1.8, −0.8, 1.2	$1–3.3 \times 10^5$
17–23	0.21–0.40	1.0	−2.8, 2.7, 1.1	7×10^4
Estimated upper limit sites/cell $<10^5$				

where K represents the apparent affinity constant for D-glucose. The smallest such figure for the Triton X-100 preparation is:

$$\frac{6 \times 10^{15}(0.5+0.23)}{1.74 \times 10^{11}} = \text{ca. } 25{,}000 \text{ sites/cell.} \quad (2)$$

Stein (1967, 1968) has advanced positive measurements on the order of 10^6 sites per cell from his data with NaI extracts. Table 4 shows a direct comparison of his equilibrium dialysis data with our own and a calculation of the number of specific D-glucose binding sites per cell. If we assume that our similarly prepared extracts did in fact include these membrane components, then we can only conclude that the upper limit of the number of binding sites is less than 10^5 sites per cell. Our final site-number estimates in Table 4 differ from Stein's by a greater factor than do our last-column figures because we used lower sugar concentrations with the more concentrated extracts and because our work was done at lower temperatures where it is recognized that the transport system functions at higher affinities (lower K's). Our estimates are in reasonable accord with the recent observations of Kahlenberg, Urman and Dolansky (1971) who estimated 1.9 to 2.5×10^5 sites per cell, using a method of differential binding dependent upon the presence of $(NH_4)_2SO_4$.

It is also surprising that none of the other extraction procedures used gave any indication of preferential D-glucose binding. This does not appear to be consistent with their proven ability to allow continued enzyme activity unless the true number of binding sites is very much smaller than our estimated ceiling or unless these sites specifically resisted extraction even in the face of 90% protein solubilization.

The basis for our inability to duplicate Stein's results is not clear. However, taking our results alone, we can only conclude that any selective binding which may occur in these preparations involves such a small number of sites that these methods cannot serve to measure it unless we can in some way achieve a far greater concentration of the binding material than has so far been possible.

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