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TECHNIQUES FOR ANALYSIS OF GLUCOSE BINDING BY HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

Two methods of measuring the binding of glucose by a component of the human erythrocyte membrane are described and compared, namely: (1) retardation of D-[^{3}H]glucose with respect to L-[^{14}C]glucose on columns of 'Celite'–ghost mixtures and (2) a new technique of ultrafiltration of a solution of D-[^{3}H]glucose, L-[^{14}C]-glucose and cell membrane extract.

The binding is shown to be closely associated with the glucose transport system by the following criteria for the identification of a facilitated diffusion system: (a) The binding is specific for D-glucose in preference to the L form. (b) Phloretin, a competitive inhibitor of the monosaccharide transport system as studied by kinetic experiments, is shown to inhibit the binding. (c) Saturation of binding is indicated at high levels of glucose.

INTRODUCTION

This report deals with the application of two techniques to the study of glucose binding by the human red-cell membrane. Recently the binding of glucose to a component of the red-cell membrane was demonstrated and a theoretical treatment and experimental procedure for a retardation method described[1,2]. A second technique, that of ultrafiltration has now been devised in order to overcome certain limitations of the first method. This new procedure is simple, economic in time, materials and labour and should prove of general application where the measurement of binding of small molecules by macromolecules is required. The alternative method serves to validate the results obtained by the first method and provides further evidence that what has been revealed previously is indeed binding and that the binding is associated with glucose transport.

THEORY AND METHODS

In both techniques, use is made of the fact that the glucose "carrier" in the human red-cell membrane, as shown by kinetic experiments[3], exhibits a specific high

affinity for D-glucose in preference to L-glucose or L-sorbose. Such a preferential binding has already been demonstrated in the case of glucose and sorbose by the retardation method referred to above. Here, a mixture of cell membranes and an inert material such as 'Celite' or DEAE-cellulose is packed into a column and a sample of a solution of the two labelled sugars, D-[^{3}H]glucose and [^{14}C]sorbose or L-[^{14}C]glucose, is put onto the column and then eluted. Specific binding by D-glucose results in a partial separation of the two sugars depending on the number of available sites, the dissociation constant for the interaction between glucose and the glucose-binding sites, and the concentration of free glucose in the sample. The radioactivity is counted in each fraction eluted and the amount of binding is measured by computing the percentage retardation. The method of calculation is given in the RESULTS.

In the ultrafiltration method, a cell membrane extract (solubilized and concentrated or freeze-dried) is mixed with a solution of L-[^{14}C]glucose and D-[^{3}H]-glucose. The mixture, generally about 1 ml, is placed inside a dialysis bag of Visking tubing which is threaded through a rubber bung at one end and knotted at the other. A piece of glass tubing is seated inside the Visking tubing and pushed into the bung to give an air-tight seal. The bung, *plus* tubing is then placed inside a buchner tube. It is also convenient to have a smaller test tube inside for collection of the ultrafiltrate. Both negative pressure from a water pump and positive pressure *via* the open glass tube can be applied. The sample collected is counted for radioactivity and compared with the counts of an ultrafiltrate from a sample of the same labelled sugar solution, omitting the membrane extract. If D-glucose is specifically bound by a membrane component then the ratio of D-glucose to L-glucose in the ultrafiltrate will be decreased compared with the control of the sugar solution without membrane. From the reduction in ratio we can find the percentage glucose bound and from the total D-glucose present the amount bound is calculated.

The distribution between free and bound glucose molecules can be analysed on the assumption that it follows a non-linear isotherm of the Langmuir form, *i.e.* the relationship $m = k\,c/(K_s + c)$ where m is the number of moles of bound glucose per ml of original packed red cells (approx. 10^{10} cells), c is the concentration of free glucose in the neighbourhood of the membrane component, while K_s is the dissociation constant for the interaction between glucose molecules and the glucose-binding sites. The constant k represents the maximum capacity of the component for binding glucose and is proportional to the number of sites per red cell.

MATERIALS

The cells used in these experiments were human erythrocytes from out-dated transfusion blood, preserved in acid–citrate–dextrose medium. Radioactive sugars were D-[^{3}H]glucose and L-[^{14}C]glucose, obtained from the Radiochemical Centre, Amersham. Unlabelled sugars were D-glucose (Analar) and L-glucose (Koch-Light).

Celite is the trade-name for a diatomaceous silica obtained from Johns-Manville, London, Hyflo supercel being of medium particle size while Celite 560 is a large particle size.

Visking tubing 8/32 inch diameter was used for ultrafiltration and 18/32 inch diameter for dialysis.

PROCEDURE

Erythrocyte ghosts were prepared by the addition of ice-cold distilled water to packed red cells from which the plasma and leucocytes had previously been removed. The proportion of water to packed cell volume was 100/1. The ghosts, sedimented by centrifugation at 2500 rev./min for 15 min followed by a second wash, were pink, having retained some residual haemoglobin.

To prepare Celite-supported columns, the ghosts were mixed with Hyflo supercel and the column packed with this mixture. The difficulties in handling this material were mainly due to the very fine particles which slowed the flow rate and tended to clog the outlets. This problem was overcome by the addition of a bottom and top layer of Celite 560. Also, the Hyflo supercel was boiled and fines removed before use so that reasonable flow rates were achieved. After packing the column, the haemoglobin was washed off with 3 mM phosphate buffer (pH 7.0) and then a small volume (1 or 2 ml) of the double-labelled sugars was applied to the top of the column and eluted with the same buffer. To study the effect of phloretin on glucose retardation the column was packed with Celite–ghost mixture and then washed with phloretin before putting on the sample of the sugars. After this assay, the inhibitor was removed by washing through with buffer and retardation again measured. The experiments were carried out at 23° unless stated otherwise.

Preparation of the material used in the ultrafiltration method was as follows. Ghost membranes were solubilized in a ten-fold volume of either 1.0 or 2.0 M NaI and after exhaustive dialysis against 3 mM phosphate buffer (pH 7.0) centrifuged at 33 000 × *g* for 1 h. Estimations of protein were made before and after this separation by the method of LOWRY *et al.*[4], as modified by OYANIA AND EAGLE[5]. The clear supernatant, which contained 70% of the total protein, was then either concentrated by ultrafiltration or lyophilized. The sediment remaining after the high-speed centrifugation was assayed for glucose binding by the retardation procedure after packing on a Celite column. Glucose binding was assayed by the ultrafiltration technique for both freeze-dried and concentrated solutions of the solubilized extract at different concentrations of extract and over a range of glucose levels.

Aliquots of the ultrafiltrate or of the fractions eluted from the columns were counted in a Packard Tri-Carb liquid-scintillation spectrometer. The solvent was a mixture of ethanol, and a scintillator of 0.4% 2,5-diphenyloxazole and 0.04% dimethyl 1,4-bis-2(5-phenyloxazolyl)benzene in a xylene base. The efficiencies of the counts were 7% for ^{3}H and 36% for ^{14}C.

RESULTS

Table I records the retardation of D-glucose with respect to L-glucose as a function of the concentration of glucose in the sample, using Celite–ghost columns. Ghost membranes in the mixture were equivalent to 10 ml packed red cells. The percentage retardation is found as follows. Total counts of both ^{3}H and ^{14}C are obtained for all the fractions, from which the average ratio of $^{3}H/^{14}C$ is found. The ^{14}C counts are now multiplied by this ratio. After this adjustment, which ensures that the areas under the two elution curves are equal, the difference in counts up to the point at which the two curves intersect is determined. This value, as a percen-

TABLE I

RETARDATION OF GLUCOSE BY CELITE–GHOST MIXTURES

Columns (0.9 cm × 27 cm), ghosts ≡ 10 ml packed cells. Each experiment was performed on a different column. Temperatures were at 23° except where indicated.

Expt. No.	*Volume of sample on the column (ml)*	*Glucose concn. (mM)*	*% Retardation*	*Glucose bound per ml packed cells (mμmoles)*
1	2	0.154	8.0	2.56
	1	1.54	12.1	18.7
	2	20	3.0	120
2	2	0.059	6.25	0.74
	2	10	2.65	53
3	1	1.54	7.1	
	1	1.54 (15°)	7.9	
	1	1.54	7.0	10.5
4	2	0.15	11	4.1
	2	0.15*	3.2	
	2	0.15	8.5	3.2
5		0.15*	4.0	
	2.2	0.15	7.3	3.1

* In the presence of $4 \cdot 10^{-5}$ M phloretin.

tage of the total counts, gives the percentage retardation. This difference (reversed in sign) is, of course, found in the trailing edge. The percentage retardation is seen in Table I to decrease with increasing concentrations of glucose indicating a limited number of sites. The third experiment in Table I illustrates that the effect of reducing the temperature to 15° is a slightly increased binding while the original retardation is restored with a return to room temperature.

The presence of $4 \cdot 10^{-5}$ M phloretin has the effect of decreasing glucose retardation (Table I, Expts. 4 and 5). The inhibition of glucose binding is seen to be reversible, removal of the phloretin largely restoring the binding.

In an experiment with the sediment obtained after high-speed centrifugation of membrane extract (equivalent to 10 ml packed cells), retardation with the Celite–sediment mixture was 1.9% for a 1-ml sample of 0.4 mM glucose or 0.76 mμmole bound per ml packed cells. This binding is only some 10 to 20% of the binding capacity of the intact cells (interpolating from the data recorded in Table I). Since the sediment comprises 30% of the total protein, its binding activity would appear to be rather less than that of the soluble portion of the extract. It is uncertain, however, precisely what proportion this is since there was a tendency for this sediment to be washed off the column during the assay.

The results of glucose binding by solubilized extract using the ultrafiltration method are tabulated in Table II. To find the percentage binding, an aliquot of the ultrafiltrate from the glucose-extract mixture inside the bag is counted for radioactivity and the ratio of ^{3}H to ^{14}C counts obtained. This ratio is compared with that of a control handled in the same way but omitting the extract. An example of the computation is given in the footnote of Table II. Table II indicates once again

TABLE II

GLUCOSE-BINDING DATA BY THE TECHNIQUE OF ULTRAFILTRATION

Volume of the mixture (ml)	*Glucose concn. (mM)*	*% Binding*	*Glucose bound per ml packed cells (mμmoles)*	*Extract concn. (ml packed cells per ml mixture)*	*k (mμmoles/ml packed cells)*
1	0.28*	8.8	8.2	3	149
1	2.8	5.9	55	3	149
1	14	2.4	111	3	149
1	0.20	15.3	5.2	5	98
1	10	3.6	72	5	98
0.8	0.20	7.0	4.0	3	120
0.8	4.0	9.0	38	9	90
0.8	4.0	9.5	20	19	45
0.8	1.0	12.7	10	9	84
0.8	1.0	7.1	3.8	19	22

* Example of the computation of percentage glucose binding:

	$\frac{^3H \text{ counts}}{^{14}C \text{ counts}}$	% Binding
Ultrafiltrate of glucose + extract	1.55	$\frac{1.70 - 1.55}{1.70} = 8.8$
Ultrafiltrate of glucose only	1.70	

that the binding capacity of the extract can be saturated at increasing concentration of sugar. The limited data of the first experiment (from a best fit to a rectangular hyperbola of three points) yields a value of $K_s = 4.8$ mM and $k = 149$ mμmoles/ml cells. For the two points in the second experiment, $K_s = 3.6$ mM and $k = 98$ mμmoles/ml cells. The maximum capacity k was computed for the remaining experiments from the amount bound at the concentrations recorded assuming a value of 5 mM for K_s, the derived values being listed in column 6 of Table II. It can be seen that there is apparently a dependence of the maximum capacity on the concentration of the extract, an increase in the concentration of extract resulting in a diminished maximum capacity.

When a saturating amount of thymol was added to the extract, no glucose binding was observed, but the presence of chloramphenicol (20 μg/ml mixture) resulted in only a slight reduction in binding.

DISCUSSION

The presence of a glucose-binding component in erythrocyte membranes has been confirmed by two methods and this binding has been shown to be closely associated with the glucose transport system by the following criteria for identification of a facilitated diffusion system[6].

(1) The binding is specific for D-glucose in preference to L-glucose as seen from the results of both retardation on columns of Celite–ghost mixtures and binding of D-glucose by solubilized extracts in the ultrafiltration method.

(2) A portion of the binding is inhibited by phloretin, a competitive inhibitor of the monosaccharide transport system as studied by kinetic experiments. Now, K_i for phloretin at 38° is $4.9 \cdot 10^{-6}$ M (ref. 7). The phloretin level in our experiments at 23° was apparently insufficient for complete inhibition so that unless the inhibitor constant increases with reduction in the temperature, it may be that there is some glucose binding not associated directly with transport.

(3) Saturation of binding is indicated at high levels of glucose. The application of the Langmuir equation to the data yields a measure of the two parameters K_s, the dissociation constant, and k, the maximum capacity of the membrane. The value of K_s obtained (about 4 mM at 23°) is in fair agreement with that found from kinetic experiments on whole cells where $K_s = 11$–13 mM, at 25° (ref. 8). Whether this difference is significant is uncertain.

The finding of a decrease in the binding capacity per cell with increasing concentrations of the membrane extract (Table II) can be interpreted in various ways. One possibility is a mutual association of the binding molecules to form aggregates with a lower capacity to bind glucose. If this case applies, extrapolation to zero concentration would provide a means of correcting for the decrease in the capacity.

A question raised in private discussion with the authors was whether the observed retardation of glucose by ghosts as previously reported[1,2] could be attributed to some residual selective permeability of the ghosts. This possibility could not be ruled out entirely at that stage although we had assumed that the ghosts in our procedure were permeable in a non-selective fashion and that the reversal of haemolysis by the addition of salts was a necessary step for the restoration of the facilitated diffusion system[9]. Since, however, the results reported in the present paper, using both ghosts and completely solubilized membrane extracts are substantially in agreement, we conclude that the retardation previously reported was due to glucose binding and that the ghosts, prepared by our method, have indeed, no detectable selective permeability.

NOTE ADDED IN PROOF (Received July 21st)

We now find substantially similar results to the above when membrane extracts together with a mixture of labelled sugars are merely dialysed against a small volume of buffer and the dialysate is sampled, a procedure of selective retardation which is yet more economic.

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