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INVESTIGATIONS ON THE EXISTENCE OF A SPECIFIC RETENTION OF D-GLUCOSE BY THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

1. The retention of sugars by human erythrocyte membranes after incubation with different solutions containing a ^{3}H- and a ^{14}C-labelled sugar was compared by liquid scintillation counting, using a three-channel procedure with which it is possible to detect a change in the channel ratio of ^{3}H and ^{14}C of 0.2 %.

2. The retention of D-glucose by fragmented white ghost cell membranes did not differ from that of L-glucose or D-galactose.

3. D-Glucose was taken up by intact pink ghost cells to a slightly greater extent than D-galactose at sugar concentrations of 1 mM or 40 mM. The increased uptake of D-glucose persisted after addition of $HgCl_2$ and phloretin in concentrations which block sugar transport.

4. It is concluded that the retention of D-glucose by pink ghost cells does not bear any resemblance to the characteristics of the sugar transport system of the human erythrocyte as derived from kinetic data. The experiments thus do not support previous results in which it has been suggested that an interaction of D-glucose with the sugar transport system of human erythrocyte membranes is detectable in binding experiments.

The movement of sugars like D-glucose, D-mannose and D-galactose across the human erythrocyte membrane occurs by a transport mechanism which exhibits the characteristics of an equilibrating carrier system[1–3]. Various attempts have been made to elucidate the molecular mechanism of the transport of these sugars[4–7]. Thus, BOBINSKI AND STEIN[6], using ghost cells immobilized on an inert support as a chromatographic column, observed that elution of D-[^{3}H]glucose was retarded relative to that of L-[^{14}C]sorbose, which has a low affinity for the transport system. It has been suggested that the retardation of D-glucose is due to an interaction with the sugar carrier in the membrane. However, a difficulty in the evaluation of the column experiments has been the uncertainty to what extent the ghost cells are readily permeable to L-sorbose. Thus, LEFEVRE AND MASIAK[8] have recently published an extensive study on the elution behaviour of columns impregnated with ghost cells, and these authors have obtained evidence for the view that the delay of D-glucose relative to that of L-sorbose is caused by intracellular transport rather than binding of D-glucose to the membrane of the ghost cells.

The purpose of the experiments presented in this report has been to resolve

the question of whether there is a specific retention of D-glucose by human erythrocyte membranes which might be taken as evidence for binding to the sugar carrier. We have tried to circumvent the problem of the possible impermeability of non-transported sugars by comparing the retention of D-glucose with that of D-galactose or of D-glucose with that of L-glucose in fragmented membrane material. Furthermore, we have measured the relative contents of D-glucose and D-galactose in intact ghost cells. The rationale for comparing retention of D-glucose and D-galactose is that kinetic analysis has revealed that the affinity of D-glucose to the transport system is one order of magnitude higher than that of D-galactose[9]. L-Glucose was only used in conjunction with fragmented membrane material since this sugar belongs to the group of non-transported sugars[10].

Experiments on fragmented membranes of human erythrocytes

White ghost cells were prepared according to the method of DODGE *et al.*[11] under sterile conditions, and the ghost cells were suspended in an isotonic electrolyte solution[12], frozen quickly in an acetone–CO_2 mixture, and thawed immediately before use. The procedure as finally evolved for carrying out the binding experiments was as follows: A small amount of radioactive stock solution (0.1 ml) containing approx. 50 μC of a ^{3}H- and 5 μC of a ^{14}C-labelled sugar, dissolved in the isotonic electrolyte fluid together with 11 mM of each of the unlabelled sugars, was added to triplicate samples of 1 ml of the freeze–thawed ghost suspension. The samples were incubated for 20 min at 4° or 37°, following which membrane material was sedimented by centrifugation at 100000 × *g* for 0.5 h, maintaining the previous incubation temperature. The supernatant was discarded, and fluid adhering to the sides of the wall was thoroughly wiped off by the aid of filter paper. The sediment was then extracted with 5% (w/v) trichloroacetic acid for 12–18 h, and the precipitated material was centrifuged off. Control samples consisting of 0.1 ml of the electrolyte solution without radioactivity and 1 ml of the freeze–thawed ghost suspension were treated in the same way. 50 μl of the electrolyte solution was added to 600 μl of the supernatant of the experimental samples, while 50 μl of a suitable dilution of the radioactive stock solution in the electrolyte buffer was added to 600 μl of the supernatant of the control samples. These manipulations were done to ensure the same quenching characteristics of experimental and control samples, *i.e.* the same concentration of trichloroacetic acid and of any quenching material that might originate from the ghost sediment. The dilution of the radioactive stock solution was determined on the basis of preliminary counting and was intended to bring the counts per min of controls within 10% of that of the experimental samples. The ratio between the disintegrations originating from ^{14}C and ^{3}H (^{14}C/^{3}H) was assessed by determinations on a Packard liquid scintillation counter (Model 3320), using a highly accurate three-channel procedure which is capable of detecting changes of ^{14}C/^{3}H of $\pm$ 0.2% (S.D.)[13].

Table I shows the results of experiments with differently labelled mixtures of D-glucose and D-galactose or D-glucose and L-glucose. It can be seen that only small changes of ^{14}C/^{3}H of 0.1–0.3% were observed with all combinations of sugars, irrespective of whether the temperature was 4° or 37°. Furthermore, the differences are all in the same direction, and therefore they cannot be attributed to specific binding of the sugars tested. Rather it would appear that the difference is due to an isotope effect, the ^{14}C-labelled sugars being taken up by the membrane material to a slightly

TABLE I

COMPARISON BETWEEN THE RETENTION OF D-GLUCOSE AND OTHER SUGARS BY FREEZE–THAWED WHITE GHOST CELLS

The experiments were done as described in the text. Triplicate determinations on each sample were carried out, and the vials were counted repeatedly until the statistical error was less than ± 0.1% (S.D.). The results in the table are the mean value obtained in three experiments. Figures in parentheses indicate the range of variation.

Sugar mixture	*Temperature* (°)	$^{14}C/^{3}H$ *of sugar mixture* ($\mu C/\mu C$)	*Change of* $^{14}C/^{3}H$ *of ghost extract* (%)
D-[^{3}H]glucose/L-[^{14}C]glucose	4	0.091	0.1 (−0.1–0.2)
	37	0.083	0.3 (0.3–0.4)
D-[^{3}H]glucose/D-[^{14}C]galactose	4	0.107	0.2 (—0.1–0.5)
	37	0.096	0.2 (—0.1–0.5)
D-[^{3}H]galactose/D-[^{14}C]glucose	4	0.087	0.2 (0.0–0.6)
	37	0.091	0.3 (0.0–0.7)

greater extent than the tritiated sugars. It should be noted that the concentration of D-glucose used in the experiments of Table I is well below that required to obtain half-maximal saturation of the transport process at 37° (refs. 9 and 14), and thus conditions for detection of differences in binding are expected to be optimal. Besides, no difference in binding was observed in a few experiments in which tracer levels (10^{-4}–10^{-5} M) of sugar were employed (not shown in the table).

Experiments on pink ghost cells

The experiments described in the previous section, in contrast to previous reports[6,7], provide no evidence for specific binding of sugars to the human erythrocyte membrane. However, it might be argued that the transport system of freeze–thawed white ghost cells is possibly lost or inactivated during the preparative procedure. We have therefore conducted another series of experiments on a pink ghost cell preparation with a documented capacity for transport of D-glucose[15]. In these experiments the removal of D-glucose and D-galactose from the extracellular fluid of the incubation mixture has been compared after equilibration of the intracellular phase with sugar.

Pink ghost cells were prepared essentially as described by LEFEVRE[15], the hemolysis taking place in a 100-fold volume of deionized water containing 1 mM D-glucose and 1 mM D-galactose. Triplicate samples of 1 ml of ghost cells suspended in the isotonic electrolyte buffer (cell volume approx. 80%) were mixed with 25 μl of a stock solution of radioactive sugars. The stock solution contained 1 mM D-glucose and 1 mM D-galactose, dissolved in the electrolyte buffer, together with either (1) approx. 50 μC D-[^{3}H]glucose and 5 μC D-[^{14}C]galactose, or (2) approx. 50 μC D-[^{3}H]-galactose and 5 μC D-[^{14}C]glucose. The ghost cells were centrifuged off after incubation of the ghost cells with radioactivity for 20 min at 25°, and 100 μl supernatant was pipetted off, treated with 5% trichloroacetic acid, and counted as described in the previous section. The supernatant of non-radioactive incubation mixtures served as a control. In some cases the removal of radioactive D-glucose and D-galactose was compared at a concentration of 40 mM of both sugars, and in these experiments a

different incubation procedure was employed to effect equilibration between the extracellular and intracellular phase as described in the legend of Table II.

It can be seen from the data of Table II that at a concentration of 1 mM of both sugars the ratio between D-[^{3}H]glucose and D-[^{14}C]galactose of the extracellular phase of the incubation mixture is 0.3 % above that of the original radioactive stock solution, whereas the ratio between D-[^{3}H]galactose and D-[^{14}C]glucose is increased by 1.5% under the same conditions. Converse changes were found by analyzing trichloroacetic acid extracts of the ghost sediment (not shown in the table). Thus the ratio between D-[^{3}H]glucose and D-[^{14}C]galactose was 0.8 % lower than that of the control solution, and the ratio between D-[^{3}H] galactose and D-[^{14}C]glucose was decreased by 1.4 %. These results indicate (1) that the content of ^{14}C-labelled sugar in the ghost cells is about 1 % higher than that of the corresponding tritiated sugar, which may be due to an isotope effect, and (2) that there is preferential uptake of D-glucose as compared to D-galactose, which accounts for the difference in the channel ratio of the differently labelled pairs of sugars. The question remains whether this extra uptake of D-glucose represents preferential binding to the sugar carrier. It can be seen from the other data of Table II that the dissimilarity in the channel ratio of the differently labelled pairs of sugars persists when $HgCl_2$ or phloretin is added to the incubation medium in concentrations which are known to arrest the transport of sugars[12,16]. Besides, a relatively pronounced difference of ^{3}H/^{14}C remains when the concentration of the sugars in the incubation mixture is raised to approx. 40 mM in spite of the fact that the transport of D-glucose is saturated under these conditions[1,3].

It must be concluded that the results obtained with pink ghost cells do not bear any resemblance to the characteristics of the transport of D-glucose as derived from kinetic experiments. Whatever may be the reason for the low degree of preferential uptake of D-glucose in pink ghost cells, it does not seem to depend on the binding

TABLE II

COMPARISON BETWEEN THE REMOVAL OF LABELLED D-GLUCOSE AND D-GALACTOSE FROM THE EXTRACELLULAR PHASE OF PINK GHOST CELL SUSPENSIONS

The experiments were carried out as described in the text. $HgCl_2$ and phloretin were added to give a final concentration of 1 mM after incubation of the pink ghost cell suspension with radioactivity for 20 min. In the experiments employing a high concentration of sugars, the ghost cell preparation was suspended in a large volume of 40 mM D-glucose and 40 mM D-galactose, dissolved in electrolyte buffer, for 4 h at 37° to effect equilibration of the intracellular phase with sugars, and the incubation period in this case was extended to 1 h. The samples were counted as described in the legend of Table I. The results of the table denote the mean of three experiments. Figures in parentheses indicate the range of variation.

Concentration of D-*glucose and* D-*galactose (mM)*	*Addition to the suspension*	*Change of* D-[^{3}*H*]*glucose/* D-[^{14}*C*]*galactose in supernatant* (%)	*Change of* D-[^{3}*H*]*galactose/* D-[^{14}*C*]*glucose in supernatant* (%)
1	None	0.3 (0.1–0.4)	1.5 (1.0–1.8)
1	$HgCl_2$	0.2 (0.0–0.7)	2.0 (1.5–2.5)
1	Phloretin	0.2 (–0.1–0.7)	2.2 (1.5–3.2)
40	None	0.0 (–0.2–0.4)	1.2 (1.0–1.5)

of D-glucose to the sugar carrier. The number of carrier sites for D-glucose transport must thus be very low, indicating a high turnover number of the D-glucose translocating system. This conclusion argues strongly against the view that D-glucose transport occurs *via* a mobile carrier dissolved in membrane lipid, as has been previously suggested[17]. Even if all the hydrophilic groups of D-glucose were "masked" by such a carrier, the molecular weight required for the formation of this complex would probably result in low turnover numbers[18]. The results therefore suggest that the pathway of D-glucose is through a hydrophilic portion of the membrane, but the mechanism of the translocation process remains obscure.

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