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EVIDENCE FOR AN INTERMEDIATE STEP IN CARRIER-MEDIATED SUGAR TRANSLOCATION ACROSS THE BRUSH BORDER MEMBRANE OF HAMSTER SMALL INTESTINE

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SUMMARY

The following observations have been made about the interactions of 6-deoxy-L-galactose with the Na^+-dependent sugar transport system of hamster small intestine.

1. 6-Deoxy-L-galactose does not appreciably enter the intracellular spaces of incubated intestinal segments and phlorizin is devoid of action on this minimal entry.

2. 6-Deoxy-L-galactose is a competitive inhibitor of the Na^+-dependent sugar transport system with a K_i of about 20 mM.

3. 6-Deoxy-L-galactose does not elicit counterflow of a known substrate, L-glucose, under conditions where counterflow is elicited by a substrate, 1,5-anhydro-D-glucitol, of approximately equivalent affinity.

4. The K_i of 6-deoxy-L-galactose increases with the reduction in Na^+ concentration as does the K_m of 3-*O*-methylglucose. Consequently, the ratio K_m 3-*O*-methylglucose/K_i 6-deoxy-L-galactose remains constant over a 5-fold range of Na^+ concentration; that is, the competitive interaction of 6-deoxy-L-galactose is Na^+-dependent.

5. 6-Deoxy-L-galactose acts like mannitol in transmural potential studies; that is, it induces a streaming potential only. It does not induce Na^+ movement despite the fact that its carrier interaction is Na^+-dependent.

From these observations the inference is drawn that mobility of the carrier–substrate complex which results in translocation of a substrate involves a non-covalently bonding transformation of the complex; a transformation which cannot take place when 6-deoxy-L-galactose is bound to the carrier. 6-Deoxy-L-galactose forms an abortive complex.

INTRODUCTION

A number of years ago, it was reported from this laboratory[1] that 6-deoxy-L-galactose (L-fucose) was not transported by the glucose active transport system of

Abbreviation: PD, potential difference.

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hamster small intestine but was, nonetheless, an inhibitor of the transport of 1,5-anhydro-D-glucitol (1-deoxyglucose), a compound which is transported by this system. The suggestion was made[1,2] that 6-deoxy-L-galactose might be able to combine with the same site or structure as actively transported sugars and their analogues but be unable to undergo subsequent steps in the process.

In the intervening years, our efforts have been concentrated on arriving at an appropriate formulation of the brush border membrane phase of active glucose transport. We postulated[2,3] that this phase was subserved by a bifunctional mobile carrier capable of combining with and transporting both substrate and Na^+, and provided experimental evidence[4-7] in support of this hypothesis. Further convincing evidence in support of this hypothesis has continued to accumulate not only in our laboratory[8,9] but also in others[10-12] and it has been extended to the active transport of compounds other than monosaccharides[13].

As is consistent with current mobile carrier concepts[14] the formulation provides only for non-covalently bonded complex formation between the substrate and the recognition site of the carrier. It is a *sine qua non* of these concepts, that the carrier is mobile in the free or the combined form. Consequently, simple theory demands that interaction of a compound with the recognition site of the carrier should lead to its transfer, unless the compound chosen introduces steric or chemical hindrances to the mobility of the complex[15].

Extensive studies and analysis of the specificity of the recognition site of the glucose active transport system of hamster intestine[15] reveal what is rather to be expected for a special case of a general phenomenon. Fundamentally, the carrier site recognizes the pyranose ring with the mutual affinity between the substrate and the carrier site being influenced by the identity and configuration of the substituents at each carbon atom in the ring. With the, so far sole, exception of 6-deoxy-L-galactose, interaction and transport of a range of monosaccharides is consistent with their respective configurational relationships to D-glucose. For example, L-glucose has been found to be actively transported with a K_m of about 65 mM (refs. 16, 17). Consequently, the behavior of 6-deoxy-L-galactose appears to be anomalous; that is, there are no immediately obvious steric or chemical reasons to account for its action as an inhibitor and its failure to be transported. We have studied this anomaly with the results recorded below.

METHODS AND MATERIALS

Incubation technique

The present experiments were performed by the technique *in vitro* of CRANE AND MANDELSTAM[16] as modified in a more recent publication[17].

Hamsters were fasted overnight. Approx. 200–300 mg wet wt., of tissue were placed in 25-ml erlenmeyer flasks, containing 5 ml of Krebs–Henseleit phosphate or bicarbonate buffer[19] with the appropriate added substrates. The buffer was gassed with pure oxygen or O_2–CO_2 (95:5, v/v) when bicarbonate buffer was used. In some experiments the buffer was modified by replacing Na^+, to the extent indicated in the graphs, by $Tris^+$. Isoosmolarity was preserved by adding substrates as a 0.3 M solution.

In K_m experiments, when the tissue was incubated at different substrate concentrations with an inhibitor, D-mannitol was added to the control in the same con-

centrations as the inhibitor. D-[^{14}C]Mannitol was also used as a marker for extracellular space[7].

In the demonstration of counterflow, two successive incubations were performed: (1) Rings of everted intestine were allowed to accumulate substrate for the time indicated and controls were assayed for the amount accumulated: (2) samples of the tissue with accumulated substrate were reincubated in fresh media containing the same substrate concentration with and without inhibitor. In experiments using more than 30 mM of inhibitor, an equal concentration of D-mannitol was added to the substrate control.

Transmural potential (PD) measurements

Suspended everted sacs, 5–6 cm in length, were prepared from the middle of the small intestine of non-fasted golden hamsters. The sacs were incubated at 37° in Krebs–Henseleit bicarbonate buffer[19] or a modified buffer which has been equilibrated with a gas mixture of O_2–CO_2 (95:5, v/v) for 1 h prior to use. $Tris^+$ was substituted for Na^+ to achieve media equal to the Krebs–Henseleit bicarbonate buffer in ionic balance, but lower in Na^+ concentration. The $Tris^+$ and Na^+ content of the media are indicated in the graphs.

In all experiments, the mucosal and serosal compartments contained the same buffer. Mucosal fluid was 10 ml. The serosal fluid volume was variable but its level was brought to just above the tissue-glass junction after positioning of the serosal salt bridge in the apparatus described below.

Water solutions of 0.3 M 3-*O*-methylglucose, 6-deoxy-L-galactose or D-mannitol were added to the mucosal fluid only. Preliminary experiments showed that for the concentrations of materials used, there was no difference in the ΔPD values whether the solvent was 0.9% NaCl, incubation buffer or distilled water.

Other preliminary studies showed that the ΔPD induced by 6-deoxy-L-galactose, in concentrations up to at least 30 mM, was negative and indistinguishable from the D-mannitol-induced negative ΔPD (Table I). The ΔPD induced by 3-*O*-methylglucose, on the other hand, was positive (Table I). In order to delete the osmotic streaming potential from the data, D-mannitol, in concentrations equal to the 6-deoxy-L-galactose concentration to be tested, was added during measurement of resting potentials and uninhibited 3-*O*-methylglucose ΔPD values. In this way, the actual ΔPD values induced by 3-*O*-methylglucose were observed.

The incubation procedure and apparatus used for the majority of the PD studies were based on the descriptions by LYON AND CRANE[9]. The incubation apparatus consisted of a No. 1 rubber stopper fitted with a glass tube used to suspend the everted sac, polyethylene tubing which supplied the gas mixture to the mucosal fluid, a mucosal salt bridge and an opening to allow for gas escape. The serosal salt bridge was inserted through the glass tube, approximately one-half way into the fluid and secured by a small cork at the top of the glass tube.

This assembly was inserted into an 18 mm × 98 mm plastic tube containing the complete incubation media, various concentrations of 3-*O*-methylglucose, 6-deoxy-L-galactose or D-mannitol. The tubes were kept in a test tube rack in a 37° waterbath and the assembly was moved from tube to tube. All PD measurements were made 30 sec after transfer to a new tube.

In one set of experiments, incubation was carried out in a 30-ml syringe instead

of a plastic tube. A No. 4 rubber stopper was fitted as decribed above, except for an additional opening used to inject the medium and sugar solutions. The incubation solutions were removed through a rubber tube connected to the bottom of the syringe. Thus, instead of moving the tissue from tube to tube, the tissue remained stationary and the medium was changed. To reduce degenerative effects associated with total fluid removal and refilling, the incubation medium with either 6-deoxy-L-galactose or D-mannitol was placed in the syringe, and 3-*O*-methylglucose was added in discrete steps of increasing concentration.

In order to measure PD, salt bridges, connecting the incubation media and electrodes, were prepared by filling PE polyethylene tubing (Clay-Adams, New York) with a warm solution of 3 M KCl–1.5 % agar (special Agar-Difco Labs., Detroit) and allowing to cool. The electrodes were prepared by coiling one end of cleaned, 18-gauge sterling silver wires (William Dixon, Newark). Silver chloride was deposited on the coiled portion of the wire. Voltage changes were detected with a Keithley 610B Electrometer (Keithley Instruments, Cleveland).

Compounds

The following compounds were from commercial sources: D-mannitol from Fisher Scientific Corp., 2-deoxy-D-glucose and phlorizin from Nutritional Biochemical Corp., 6-deoxy-L-galactose from Pfanstiehl Laboratories, 3-*O*-methyl-D-glucose from Calbiochem, L-glucose from Sigma Chemical Corp. Radioactive compounds were D-[^{14}C]mannitol, 2-deoxy-D-[^{14}C]glucose, L-[^{14}C]glucose from New England Nuclear Corp., 6-deoxy-L-[^{14}C]galactose and 3-*O*-methyl-D-[^{14}C]glucose from Calbiochem. 1,5-Anhydro-D-[^{3}H]glucitol and 1,5-anhydro-D-glucitol were prepared in our laboratory as described in an earlier publication[18]. The purity of L-glucose and 6-deoxy-L-galactose was confirmed by gas chromatography (Beckman GC 4) following silylation by a method similar to that of SWEELEY *et al.*[20] using 6-ft columns with PO-1 packing at 140 and 160°.

Analytical methods

Incubations were terminated by removal of the tissue. Tissue and media were processed for assay as described by CRANE AND MANDELSTAM[18].

D-[^{14}C]Mannitol, 2-deoxy-D-[^{14}C]glucose, 6-deoxy-L-[^{14}C]galactose, 3-*O*-methyl-D-[^{14}C]glucose, 1,5-anhydro-D-[^{3}H]glucitol and L-[^{14}C]glucose were assayed with the Beckman Liquid Scintillation System.

Calculation of uptake data

Results are expressed as rates of entry in mmoles of substrate accumulated per ml of tissue water in a given time, assuming a water content of approx. 80 % of the tissue weight[18]. All data in K_m studies were corrected for the D-mannitol space.

$$\text{Percent filling} = 100 \times \frac{\text{mmoles/ml tissue water}}{\text{mmoles/ml medium}}$$

The results in tables and figures are averages of duplicates or triplicates.

Transmural potential data

Everted sacs from three individual animals were used to obtain the data at each medium-Na^+ concentration. Also, three trials at the same Na^+ level were per-

formed with each sac. Data variation within a single preparation was minimal and often no difference was observed. Since the pooled data from the three animals were not normalized, the standard deviations of the reported means were larger than the individual sac variations.

In the Lineweaver–Burk plots, the results are expressed as means ± S.D. The coefficient of variation for individual points ranged from 0–43 %. The curves were calculated by computer, programmed to determine a regression line by the least-squares method. K_m and K_i values were obtained from the computer plots and are reported in the tables.

RESULTS

Failure of transport of 6-deoxy-L-galactose

Rings of everted hamster small intestine were incubated for 1 h with 6-deoxy-L-galactose. As can be seen in Fig. 1, after as much as 60 min of incubation, 6-deoxy-L-galactose entry into the tissue minimally exceeded the value obtained for the D-mannitol used as a marker for extracellular space. In contrast, the non-actively transported sugar, 2-deoxy-D-glucose, achieved a far higher degree of entry in the same time period. Phlorizin, at a concentration completely inhibitory to glucose active transport under similar conditions[21] had no effect on entry of these compounds.

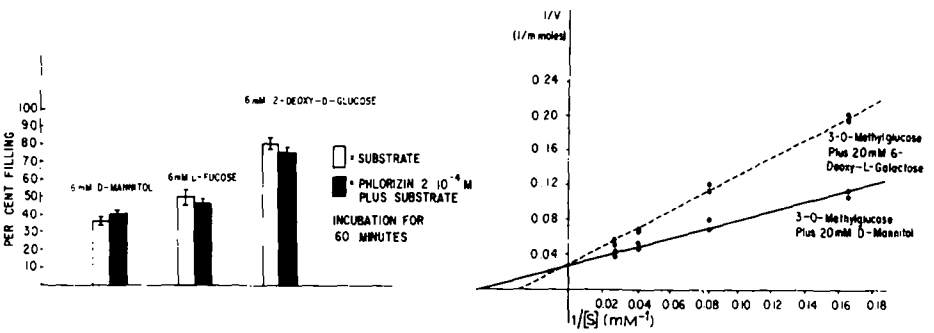

Fig. 1. Uptake of D-mannitol, 6-deoxy-L-galactose and 2-deoxy-D-glucose in the presence and absence of phlorizin. Rings of hamster small intestine were incubated in Krebs–Henseleit phosphate buffer for 60 min.

Fig. 2. Lineweaver–Burk plot of 6-deoxy-L-galactose inhibition of 3-*O*-methylglucose uptake. Rings of hamster small intestine were incubated in Krebs–Henseleit bicarbonate buffer for 15 min.

Determination of K_i of 6-deoxy-L-galactose by tissue accumulation

In earlier experiments, it was shown that 6-deoxy-L-galactose competitively inhibited the transport of L-glucose[17]. Similar results were obtained when 1,5-anhydro-D-glucitol or 3-*O*-methylglucose (Fig. 2) were used as substrates. In additional experiments, different concentrations of inhibitor were incubated with a constant concentration of 1,5-anhydro-D-glucitol and the results were plotted by the method of DIXON AND WEBB[22], as shown in Fig. 3. The intercepts yielded a K_i of about 20 mM for 6-deoxy-L-galactose, which is well within the range of K_m of several well-transported compounds which use the D-glucose pathway.

Effect of 6-deoxy-L-galactose on transmural PD

As has been demonstrated many times[9,23], actively transported sugars induce a rise in transmural PD which is accounted for [24] by the formulation of a bifunctional Na^+-dependent carrier in the brush border membrane[3,4]. Some typical results are

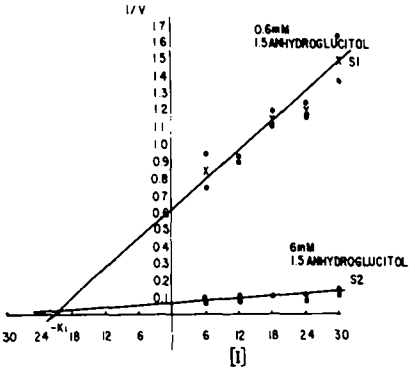

Fig. 3. Determination of inhibitor constant (K_i) of 6-deoxy-L-galactose *vs.* 1,5-anhydroglucitol by the Dixon plot. Rings of hamster small intestine were incubated for 10 min in Krebs–Henseleit phosphate buffer.

shown in Table I. Also shown in this table are data with 6-deoxy-L-galactose and D-mannitol. 6-Deoxy-L-galactose acted in a manner quantitatively equal to D-mannitol; neither compound induced a rise in PD. On the contrary, both induced a fall in PD which increased with increasing mucosal concentrations of 6-deoxy-L-galactose or D-mannitol. This kind of result has been interpreted as an osmotic streaming potential[23].

Na^+ dependence of 6-deoxy-L-galactose interaction

It has been shown by PD measurements similar to those used here that the apparent K_m of sugars binding to the common sugar carrier is Na^+-dependent[9] in the same way as when measured by sugar uptake[8]. Lowering the Na^+ concentration in the incubation medium increases not only the K_m of a substrate but also the K_i of a competitive inhibitor and to the same extent, thus keeping the ratio of K_m/K_i unchanged. This has been shown by LYON[25] for the interaction of 1,5-anhydro-D-glucitol and phlorizin using transmural PD measurements.

Inasmuch as we could not directly demonstrate Na^+-dependency of the interaction of 6-deoxy-L-galactose with the carrier because it did not induce a positive PD, we investigated this parameter by measuring the K_i of 6-deoxy-L-galactose against the K_m of 3-*O*-methylglucose, at different Na^+ concentrations using PD measurements as the most sensitive available tool. 3-*O*-Methylglucose was chosen because its K_m is reasonably close to the K_i of 6-deoxy-L-galactose as obtained by the tissue accumulation (Fig. 3). Should the binding of both compounds, 3-*O*-methylglucose and 6-deoxy-L-galactose, to the common sugar carrier be Na^+-dependent to the same extent, then the K_m of 3-*O*-methylglucose should increase at lower Na^+ concentrations to the same extent as the K_i of 6-deoxy-L-galactose.

Table II shows the results of experiments on this point. Over a 5-fold difference in Na^+ concentration, which is about the working limit to obtain easily measurable PD differences, the ratio, K_m/K_i, remained constant. It would appear that interaction

TABLE I

EFFECT OF VARIOUS SUGARS AND D-MANNITOL ON THE TRANSMURAL PD IN HAMSTER SMALL INTESTINE

PD values were obtained 30 sec after transfer and are averages of three of four experiments. The number of tests is given in parentheses.

Substrate	*Concn. (mM)*	*Substrate induced (mV) PD*
D-Glucose (4)	1	+5.5
	5	+9.6
	10	+10.1
1,5-Anhydro-D-glucitol (3)	1	+1.8
	5	−4.8
	10	−7.2
3-*O*-Methylglucose (4)	10	+5.6*
	20	+7.0*
	30	+8.1*
L-Glucose (3)	5	+0.3*
	10	+0.5*
	20	+0.9*
6-Deoxy-L-galactose (3)	10	−1.2**
	20	−2.1**
	30	−3.8**
D-Mannitol (3)	10	−1.0
	20	−2.2
	30	−3.5

* Corrected for D-mannitol osmotic streaming potential.
** Not corrected for D-mannitol osmotic streaming potential.

TABLE II

INFLUENCE OF DIFFERENT MEDIUM-Na^+ CONCENTRATIONS ON K_m OF 3-*O*-METHYLGLUCOSE AND K_i OF 6-DEOXY-L-GALACTOSE DETERMINED BY MEASUREMENTS OF TRANSMURAL PD IN HAMSTER SMALL INTESTINE

PD values were recorded in Krebs–Henseleit bicarbonate buffer 30 sec after transfer.

Na^+ concn. (mequiv/l)	*$Tris^+$ concn. (mequiv/l)*	*K_m of 3-O-methyl-glucose (mM)*	*K_i of 6-deoxy-L-galactose (mM)*	*Ratio K_m/K_i**
145	—	22	8.5	2.6
85	60	25	10.5	2.4
40	105	39	17	2.3
31	114	66	24	2.7

* Mean ± S.D. = 2.50 ± 0.22.

with the common sugar-binding site is Na^+-dependent to the same extent for both 3-*O*-methylglucose and 6-deoxy-L-galactose. We have no theoretical explanation for the difference in K_i for 6-deoxy-L-galactose when measured against 3-*O*-methylglucose ΔPD and when measured against 3-*O*-methylglucose or 1,5-anhydro-D-glucitol in tissue accumulation.

Counterflow experiments

The mobile carrier concept predicts that substrates using the same carrier will undergo the phenomenon of countertransport[14], a phenomenon which has been observed for intestinal sugar transport[26]. Countertransport means that after accumu-

lation of a substrate in the tissue, addition of a second substrate to the medium will induce an outflow from the tissue of the previously accumulated first substrate against a concentration gradient. This phenomenon requires that the second substrate enters the cell by the same carrier system as the first. If the second compound is not a substrate, but merely an inhibitor reacting with the carrier recognition site only, and not being transported, the rate of accumulation of the first compound would be expected to be reduced because of the fewer carrier sites available to it. However, counterflow would not be expected to be induced.

Counterflow experiments with 6-deoxy-L-galactose as elicitor have been carried out with L-glucose as substrate. L-Glucose was chosen because it has a relatively low affinity for the carrier recognition site and should consequently be readily inhibited. Counterflow of L-glucose induced by D-glucose has been shown previously[17].

Fig. 4 shows that 6-deoxy-L-galactose nearly completely inhibits further uptake of L-glucose, but is not able to induce an outflow of L-glucose against a concentration gradient. By way of contrast, the actively-transported compound, 1,5-anhydro-D-glucitol, which has an apparent K_m for transport of 14 mM (ref. 26), very similar in magnitude to the measured K_i for 6-deoxy-L-galactose, did induce an outflow against a concentration gradient.

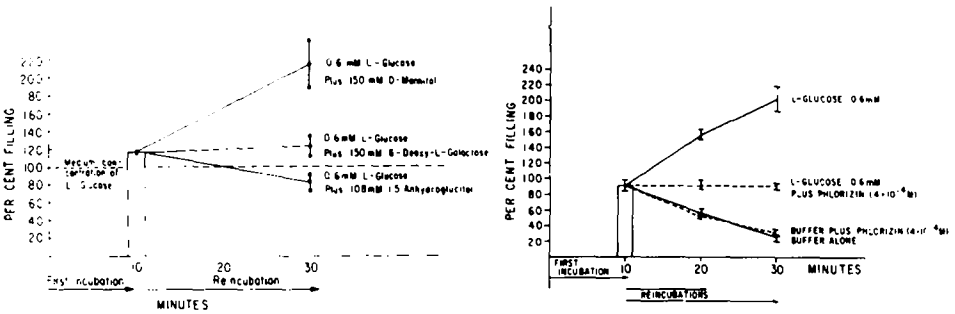

Fig. 4. Tests for counter transport of L-glucose in the presence of 6-deoxy-L-galactose or 1,5-anhydro-D-glucitol. Rings of hamster small intestine were incubated for 10 min with 0.6 mM L-[^{14}C]-glucose in Krebs–Henseleit phosphate buffer and then transferred to either 0.6 mM L-[^{14}C]glucose *plus* 150 mM 6-deoxy-L-galactose or 108 mM 1,5-anhydro-D-glucitol. Incubation was continued for an additional 20 min. During the reincubation, 150 mM D-mannitol was added to 0.6 mM L-glucose when no inhibitor was present.

Fig. 5. Failure of phlorizin to induce counterflow of L-glucose. Rings of hamster small intestine were incubated for 10 min in Krebs–Henseleit phosphate buffer with 0.6 mM L-[^{14}C]glucose and then transferred to either 0.6 mM L-[^{14}C]glucose, 0.6 mM L-[^{14}C]glucose *plus* 0.4 mM phlorizin, buffer *plus* 0.4 mM phlorizin, or buffer alone. Incubation was continued for an additional 10 or 20 min.

Since phlorizin behaves similarly to 6-deoxy-L-galactose; that is, it competes for the common sugar binding site[21] but does not enter the cell[27], we tested whether phlorizin would be able to induce counterflow. Again, L-glucose was used as a substrate. As can be seen in Fig. 5, phlorizin, at 0.4 mM, completely blocked further uptake of L-glucose, but it did not induce counterflow of L-glucose; neither did it accelerate outflow of L-glucose down its concentration gradient.

In a similar experiment, using the phenylglucoside, arbutin, as the substrate, phlorizin also only inhibited further uptake and did not induce counterflow (unpublished results).

DISCUSSION

In a continuation of our earlier observations which showed that 6-deoxy-L-galactose inhibited entry of actively transported compounds into the intestinal epithelial cell of hamster small intestine[1], kinetic studies have now revealed that 6-deoxy-L-galactose acts as a competitive inhibitor at the common sugar binding site. 6-Deoxy-L-galactose, however, does not enter the cells at an appreciable rate, as would have been expected from this interaction. In fact, from our data, it appears doubtful whether 6-deoxy-L-galactose enters the intact epithelial cell at all. The 12 % filling over the D-mannitol space achieved by 6-deoxy-L-galactose was not affected by phlorizin and may be simply a matter of adsorption of 6-deoxy-L-galactose to mucosal membrane or binding to the carrier recognition site at the mucosal brush border surface.

Additional evidence that 6-deoxy-L-galactose does not enter the cell, at least by the Na^+-dependent D-glucose pathway, was obtained by its failure to cause an increase in transmural PD. 6-Deoxy-L-galactose induced a negative PD in magnitude equal to D-mannitol, at all concentrations tested. We interpret this negative potential of 6-deoxy-L-galactose or D-mannitol as a streaming potential produced by the flow of solution through a charged membrane as a result of the osmotic pressure differences created at the mucosal side by the addition of a non-penetrating compound.

Experiments of the effect of Na^+ on the transport of substrates and the K_t of 6-deoxy-L-galactose showed that the K_t of 6-deoxy-L-galactose increased to the same extent as the K_m of the substrate when the Na^+ concentration was lowered in the medium. This means that the interaction of 6-deoxy-L-galactose with the common sugar binding site is Na^+-dependent, and does not differ qualitatively from the interaction of a transported substrate.

Phlorizin behaves similarly to 6-deoxy-L-galactose in that is competes with substrates for the common binding site[21], but does not enter the epithelial cell[27], at least at an appreciable rate. In the case of phlorizin, this anomalous behavior may be rationalized in terms of a second binding site for the aglycone portion of the

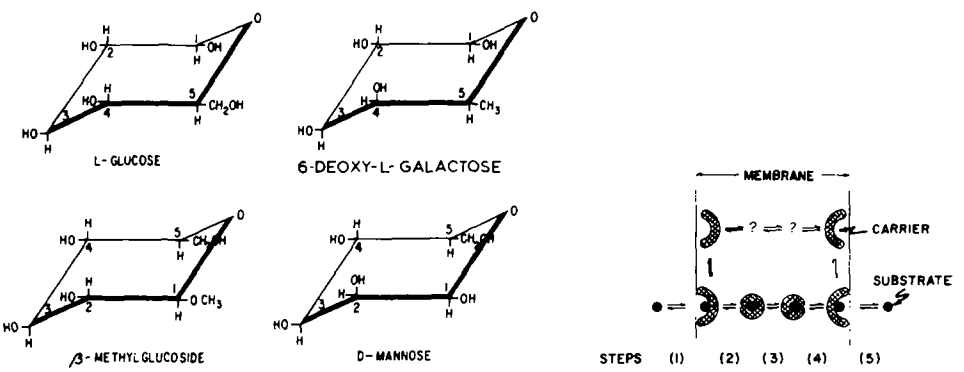

Fig. 6. The configurational relationships of 6-deoxy-L-galactose and some related sugars of special interest.

Fig. 7. Diagrammatic representation of the steps in sugar translocation. Na^+ has been omitted for clarity.

molecule which is independent and not associated with the mobile carrier[28]. An equivalent explanation is not available for 6-deoxy-L-galactose. There is no obvious steric or chemical reason why this compound should not undergo transport. Compared to L-glucose (see Fig. 6) it differs only in the C-4 hydroxyl group and the absence of an hydroxyl function at C-6. Since β-methyl-D-glucoside is well transported, the absence of an hydroxyl function at C-6 should not confer special properties to 6-deoxy-L-galactose. Mannose is not well transported, but then neither is it a good inhibitor of transport.

We do not have a final answer to the puzzling behavior of 6-deoxy-L-galactose, but our collected results lead us to propose that 6-deoxy-L-galactose binds to the recognition site of the Na^+-dependent sugar carrier to form a complex, but that the 6-deoxy-L-galactose–carrier complex cannot undergo some conformational change required for the transport of the substrate-loaded carrier through the membrane. 6-Deoxy-L-galactose forms an abortive complex. This proposal is diagrammed in Fig. 7. Five steps are indicated for the overall process. Step 1 is reversible association of the substrate with the carrier recognition site. It is assumed to be symmetrical with Step 5. Step 2 is the proposed conformational rearrangement. It is assumed to be symmetrical with Step 4. Step 3 represents translocation; that is, translational movement of the rearranged carrier–substrate complex. However, there is no way yet of knowing whether Step 3 is actually independent of and separate from Steps 2 and 4. Formulations can be imagined in which the conformational rearrangements of Steps 2 and 4 accomplish translocation.

It is of special interest to us to note that the basis for and the actual formulation of events as Steps 1 and 2 in Fig. 7 are very similar to our previous proposal for steps of substrate–enzyme interaction prior to transfer of the phosphoryl group in the brain hexokinase reaction[29].

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REFERENCES

1 R. K. CRANE, *Biochim. Biophys. Acta*, 45 (1960) 477.
2 R. K. CRANE AND W. F. CASPARY, in W. MCD. ARMSTRONG, *Intern. Symp. on Intestinal Transport of Electrolytes, Amino Acids and Sugars, Indianapolis, Ind. 1968*, C. C. Thomas, Springfield, Ill., in the press.
3 R. K. CRANE, *Federation Proc.*, 21 (1962) 891.
4 R. K. CRANE, D. MILLER AND I. BIHLER, in A. KLEINZELLER AND A. KOTYK, *Membrane Transport and Metabolism*, Academic Press, New York, 1961, p. 439.
5 J. BOSACKOVA AND R. K. CRANE, *Biochim. Biophys. Acta*, 102 (1965) 423.
6 J. BOSACKOVA AND R. K. CRANE, *Biochim. Biophys. Acta*, 102 (1965) 436.
7 I. BIHLER AND R. K. CRANE, *Biochim. Biophys. Acta*, 59 (1962) 78.
8 R. K. CRANE, G. FORSTNER AND A. EICHHOLZ, *Biochim. Biophys. Acta*, 109 (1965) 467.
9 I. LYON AND R. K. CRANE, *Biochim. Biophys. Acta*, 112 (1966) 278.
10 P. F. CURRAN AND S. G. SCHULTZ, in C. F. CODE, *Handbook of Physiology*, Section 6, Vol. 3, Alimentary Canal, Am. Physiol. Soc., Washington, D.C., 1968, p. 1217.
11 P. F. CURRAN, *Federation Proc.*, 24 (1965) 993.
12 P. F. CURRAN, S. G. SCHULTZ, R. A. CHEZ AND R. E. FUISZ, *J. Gen. Physiol.*, 50 (1967) 1261.
13 A. M. GOLDNER, S. G. SCHULTZ AND P. F. CURRAN, *J. Gen. Physiol.*, 59 (1969) 362.

14 W. F. WIDDAS, in F. DICKENS, P. J. RANDLE AND W. J. WHELAN, *Carbohydrate Metabolism and its Disorders*, Vol 1, Academic Press, London–New York, 1968, p. 1.
15 R. K. CRANE, in C. F. CODE, *Handbook of Physiology*, Section 6, Vol. 3, Alimentary Canal, Am. Physiol. Soc., Washington, D.C., 1968, p. 1323.
16 R. J. NEALE AND G. WISEMAN, *Nature*, 218 (1968) 473.
17 W. F. CASPARY AND R. K. CRANE, *Biochim. Biophys. Acta*, 163 (1968) 395.
18 R. K. CRANE AND P. MANDELSTAM, *Biochim. Biophys Acta*, 45 (1960) 460.
19 H. A. KREBS AND K. HENSELEIT, *Z. Physiol. Chem.*, 210 (1932) 33.
20 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2495.
21 F. ALVARADO, *Biochim. Biophys. Acta*, 135 (1967) 483.
22 M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, New York, 2nd Ed., 1964, p. 329.
23 R. J. C. BARRY, S. DIKSTEIN, J. MATTHEWS, D. H. SMYTH AND E. M. WRIGHT, *J. Physiol. London*, 171 (1964) 316.
24 S. G. SCHULTZ AND R. ZALUSKY, *J. Gen. Physiol.*, 47 (1964) 567.
25 I. LYON, *Biochim. Biophys. Acta*, 135 (1967) 496.
26 F. ALVARADO, *Biochim. Biophys. Acta*, 112 (1966) 292.
27 C. E. STERLING, *J. Cell Biol.*, 35 (1968) 605.
28 F. ALVARADO, *Biochim. Biophys. Acta.*, 135 (1967) 483.
29 R. K. CRANE, in P. D. BOYER, H. A. LARDY AND K. MYRBACK, *The Enzymes*, Vol.6, Academic Press, New York, 2nd Ed. 1962, p. 47.