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STUDIES ON TRANSMURAL POTENTIALS *IN VITRO* IN RELATION TO INTESTINAL ABSORPTION

IV. PHLORIZIN–SUGAR INTERACTIONS IN RAT GUT*

IRVING LYON
Department of Biochemistry, The Chicago Medical School, Chicago, Ill. (U.S.A.)

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SUMMARY

A simple and sensitive system is described for the measurement of short-circuit current, I_{sc}, across the wall of everted sacs of rat small intestine.

With this system phlorizin-dependent changes in current, ΔI_{sc}, and in transmural potential, ΔPD, have been measured and appear to be saturable functions of the phlorizin concentration in the medium. These changes in I_{sc} and PD, by analogy with similar saturation effects upon ΔI_{sc} and ΔPD induced by actively transported sugars, may be interpreted in terms of an interaction of Na^+ and phlorizin with a mobile carrier in the brush border membrane of the intestinal epithelial cell. By extrapolation from plots of $1/\Delta$PD or $1/\Delta I_{sc}$ *vs.* 1/phlorizin or sugar concentration, apparent K_m and K_i values for phlorizin, the latter determined against glucose and several of its analogs, have been estimated. The differential effects of the Na^+ concentration in the medium upon the apparent K_m and K_i values suggest that phlorizin may enter the cell by two modes of interaction with the carrier. The possible nature of these modes is discussed.

The ratio of apparent K_m for a given sugar to the apparent K_i of phlorizin against that sugar remains essentially unchanged irrespective of the Na^+ concentration. Furthermore, the K_m/K_i ratios for two or more structurally related sugars seem to allow for sharper discrimination between their affinities for the carrier than can be inferred from sugar apparent K_m values alone.

INTRODUCTION

The results of several studies lend support to the view that phlorizin enters

Abbreviation: PD, potential difference.

* Preliminary reports of this work were presented at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology held in Atlantic City, N.J., U.S.A., April 11–16, 1966 and at the Second International Biophysics Congress held in Vienna, Austria, September 5–9, 1966 (refs. 1 and 2).

the intestinal epithelial cell through the sugar active transport process even though the presence of intracellular phlorizin has not been detected. For instance, phlorizin has been shown by ALVARADO AND CRANE[3,4] to be a very effective competitive inhibitor of sugar transport by hamster intestine, *in vitro*, with an apparent K_i some 1000-fold less than the apparent K_m values of several actively transported sugars. These and other results with related phenylglycosides were interpreted as indicating that "phlorizin inhibition of active sugar transport is probably an extreme expression of the ordinary affinity and specificity characteristics of the active sugar transport process"[4]. Moreover, countertransport of sugar elicited by phlorizin has been demonstrated indicating that the carrier is not immobilized by this glycoside[5]. Recently, in high-resolution autoradiography studies with tritiated phlorizin, STIRLING AND KINTER[6] found the accumulation of label in the brush border membrane of hamster intestine suggesting interaction of phlorizin with sugar transport sites.

The findings with phlorizin cited above are certainly consistent with the carrier hypothesis of WILBRANDT AND ROSENBERG[7] according to which any substance that competitively inhibits the active transport of a given class of substrates, such as sugars, must itself be considered a potential substrate for the transport system. Furthermore, as pointed out by WILBRANDT AND ROSENBERG[7], substances having a very low K_m value may be expected to quickly saturate the carrier at the inner face of the membrane so that the maximal efflux rate may be attained at very low concentrations within the cell and, as a consequence, it may not be possible to observe accumulation.

The present studies indicate that phlorizin, just as actively transported sugars, induces an increment in the potential difference, ΔPD, and in the short-circuit current, ΔI_{sc}, and these changes appear to be saturable functions of the concentration of phlorizin in the mucosal medium[1]. In the absence of sugars, apparent K_m values for phlorizin have been evaluated by extrapolation from double-reciprocal plots of the data[1]; in the presence of glucose and several of its analogs, apparent K_i values for phlorizin have been similarly determined[1,2]. Phlorizin K_m and K_i values appear to be differentially affected by the concentration of Na^+ in the medium and the possible implications of these observations are discussed. Finally, phlorizin–sugar interactions, expressed as the ratio of apparent K_m for a particular sugar to the apparent K_i for phlorizin against that sugar, suggest that this glycoside may be used as a powerful chemical discriminator in assessing the effect of alterations in the structure of a sugar molecule upon its affinity for the sugar-binding site of the carrier[2].

METHODS

Incubation media

Everted sacs[8] were incubated at 37° in KREBS–HENSELEIT bicarbonate buffer[9] or in Tris bicarbonate buffers (25 mM) containing different amounts of Tris chloride, in isosmolar replacement for NaCl, *plus* phlorizin with or without various sugars[10]. Phlorizin solutions were prepared by dissolving a weighed amount of the crystalline glycoside dihydrate* in 2 ml of absolute ethanol and diluting to a final volume of

* Recrystallized once from hot water; the crystals were then washed 5 times with ice-cold water and dried over $CaCl_2$. A sample of this preparation appeared as a single spot when examined by paper chromatography using butanol–acetic acid–water (4:1:3, by vol.).

100 ml with the particular buffer to be used. The clear solution was shaken vigorously before use and immediately pipetted into the required volume of buffer or sugar solution. The latter solutions were also made up in the appropriate buffer. Control solutions contained D-mannitol, in comparable concentrations, to correct for osmotic disequilibria.

Tissue preparations

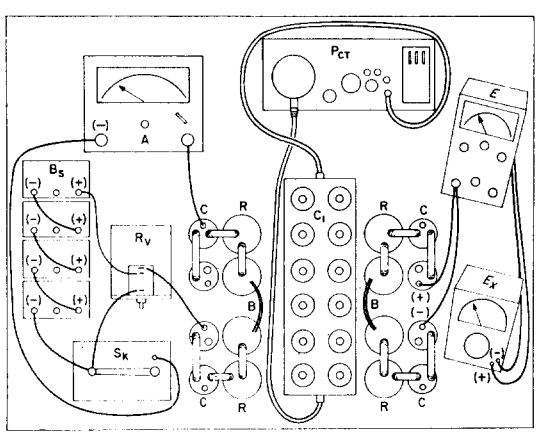
Everted sacs[8] were prepared, as previously described[10], from Sprague–Dawley MRC rats of either sex, weighing 150 ± 20 g, after a fast of 48 h during which water was available *ad libitum*.

Incubation apparatus and measurement of transmural potentials

The apparatus and the method of measurement of PD values have been described in detail in the first report of this series[10].

Measurement of short-circuit current

A diagram of the incubation apparatus and instrumentation used for the measurement of PD and I_{sc} values is shown in Fig. 1. The incubation chamber (C_1) into which incubation tubes are inserted, is maintained at 37° by a Haake type F constant-temperature circulating pump (P_{ct}). PD is read on a Keithley model 610B electrometer (E) using a Triplett model 630 voltohm meter (E_x) as an expanded scale indicator. Saturated calomel half-cells (C), balanced to zero potential, are



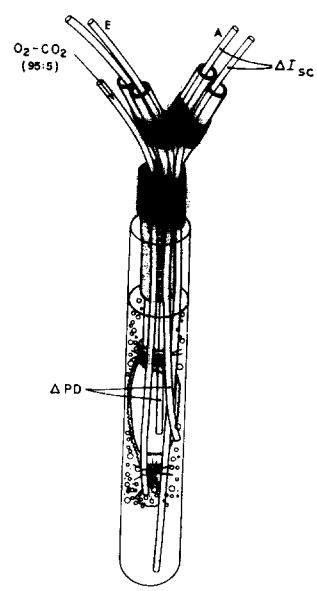


Fig. 1. Diagram of the incubation apparatus and instrumentation used for the measurement of PD and I_{sc} values. For explanation of symbols, see text.

Fig. 2. Diagram of an incubation tube containing Y-assembly with attached gut sac. The assembly is provided with mucosal and serosal bridges for ΔI_{sc} measurements and is equipped with a gas-inlet tube for aeration of the mucosal medium.

brought into contact with the tissue by means of KCl reservoirs (R) and KCl–agar bridges (B). A similar system of half-cells, reservoirs and bridges is used for I_{sc} measurements. External current to suppress the spontaneous PD to zero is supplied by Burgess 45-V "B" batteries connected in series (B_s) and a Bourns 250 kΩ variable resistance (R_v). I_{sc} may be read on a Weston model 622 d.c. microammeter (A) when the circuit is completed by depressing the key switch (S_k).

The incubation tubes (Fig. 2) containing the mucosal bathing medium are permanently set into the incubation chamber while the Y-assembly with attached gut sac is easily and quickly transferred from tube to tube. The assembly is provided with mucosal and serosal bridges for ΔPD and ΔI_{sc} measurements and is equipped with a gas-inlet tube for aeration of the mucosal medium.

Statistical variation of the data

The data reported here represent mean values; the standard deviations in percent for all values ranged from $\pm$ 7% to $\pm$ 21%.

RESULTS AND DISCUSSION

Tissue conductance

The relationship between PD and external current (Fig. 3) indicates that the tissue behaves as a linear resistor. With each change in current the PD immediately

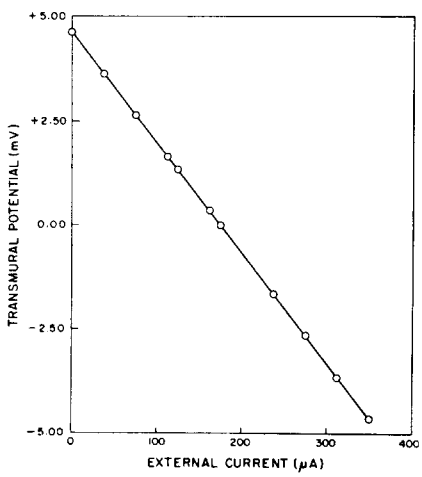


Fig. 3. The relationship between PD and external current determined in paired everted jejunal sacs.

assumed a new stable value. This linearity between voltage and current was observed in buffers with different Na^+ concentrations, both in the absence and presence of sugar or phlorizin either when added separately or together to the mucosal medium (for example, see Table I). Under these various conditions, the average tissue conductance, $\Delta I_{sc}/\Delta$PD, ranged from about 15 mmhos·cm^{-2} at a Na^+ concentration of 24 mequiv to about 25 mmhos·cm^{-2} at 145 mequiv. These values compare very favorably with those reported for rabbit ileum[11].

TABLE I

INFLUENCE OF PHLORIZIN ON TISSUE CONDUCTANCE IN RAT JEJUNUM

Paired sacs were incubated in KREBS–HENSELEIT bicarbonate buffer (pH 7.4) at 37° with graded concentrations of phlorizin in the mucosal bathing medium.

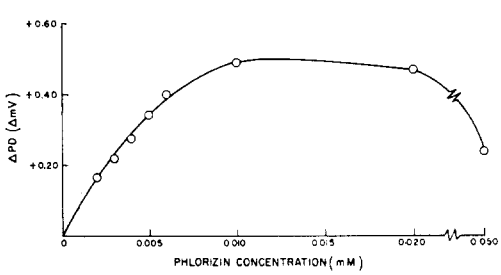
Phlorizin ($M \times 10^{-5}$)	ΔI_{sc} ($\Delta\mu A$)	ΔPD (ΔmV)	*Conductance* ($\Delta I_{sc}/\Delta PD$) ($mmhos \cdot cm^{-2}$)*
Buffer	129	2.63	20.8 ± 4.4
0.4	6.5	0.11	25.0
1.0	20.5	0.38	22.9
2.0	27.0	0.46	23.1
5.0	32.5	0.52	26.5
10.0	37.0	0.57	27.5
20.0	31.0	0.45	29.2
		Mean ± S.D.	25.7 ± 2.5

* Area $_{sac} \approx$ Area$_{cylinder} = \pi dh = (3.14)\,(0.5)\,(1.5 \pm 0.1) = 2.36 \pm 0.15\ cm^2$.

Effect of phlorizin on PD and I_{sc}

Increments in PD and I_{sc} were observed in the presence of phlorizin (Fig. 4) and these changes appear to be saturable functions of the concentration of phlorizin in the mucosal medium. Above the respective saturation concentrations of phlorizin, about 10^{-4} M in jejunum and 10^{-5} M in ileum, ΔPD and ΔI_{sc} values fell suggesting the influence of non-specific inhibitory effects.

In both jejunum and ileum ΔI_{sc} appeared to be related to ΔPD by a constant, *i.e.*, tissue conductance remained unchanged (Fig. 3, Table I). However, jejunal conductance values tend to be somewhat higher than the corresponding ileal values. Since it was previously shown that both PD and I_{sc} are linear functions of the Na^+ concentration in the medium[10–13], these differences in conductance may indicate



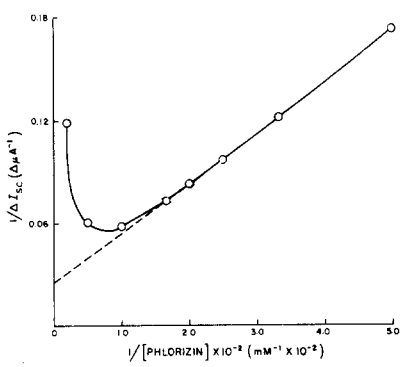


Fig. 4. The effect of graded concentrations of phlorizin, between $2 \cdot 10^{-3}$ and $5 \cdot 10^{-2}$ mM on ΔPD values in paired everted ileal sacs incubated in KREBS–HENSELEIT bicarbonate buffer.

Fig. 5. Double-reciprocal plot of $1/\Delta I_{sc}$ *vs.* 1/phlorizin concentration indicating apparent conformity to Michaelis–Menten kinetics except at higher concentrations of the glycoside. Experimental values were obtained with paired everted ileal sacs incubated in KREBS–HENSELEIT bicarbonate buffer.

jejunal–ileal differences in stoichiometry for the interaction of carrier with Na^+ and phlorizin. It is also possible that these differences may reflect the greater surface of the jejunum in accordance with the relationship between conductivity and area: $G = f(A)$.

In these studies it has been assumed that PD and I_{sc} are functions of energy-dependent Na^+ translocation across the basal membrane of the epithelial cell. Although Na^+ entry at the mucosal membrane may exceed this*, ΔPD and ΔI_{sc} have been taken as a reflection of phlorizin-dependent entry of Na^+. Lineweaver–Burk plots are suggestive of a saturation phenomenon (Fig. 5) which, by analogy with actively transported sugars[10,15,16], may be interpreted in terms of a ternary interaction of Na^+, phlorizin and a mobile carrier in the brush border membrane. Apparent K_m values have been estimated by extrapolation from the reciprocal plots (Table II).

TABLE II

INFLUENCE OF Na^+ CONCENTRATION ON APPARENT K_m AND K_i VALUES FOR THE INTERACTION OF PHLORIZIN WITH THE CARRIER IN RAT JEJUNUM

Na^+ concn. (mequiv.)	*K_m ($M \times 10^{-6}$)*	*K_i* ($M \times 10^{-6}$)*
145	6.51	0.53
120	5.83**	2.30
24	3.18	11.8

* Determined against glucose.
** Interpolated value.

Phlorizin inhibition of sugar entry

If phlorizin competes with sugar for a site on the carrier[1–6], then, according to the carrier concept[7], the K_m for phlorizin should be expected to approach its K_i value determined in the presence of an actively transported sugar. Measurements of ΔPD and ΔI_{sc} were obtained with paired jejunal and ileal sacs incubated in KREBS–HENSELEIT bicarbonate buffer containing graded concentrations of various sugars, both in the absence and in the presence of phlorizin, and typical results are shown in Fig. 6. Analysis of these data indicate, as has been shown previously in sugar accumulation studies[3,4], that phlorizin is a competitive inhibitor of the sugar-dependent PD and I_{sc} (Fig. 7).

Influence of Na^+ on apparent K_m and K_i values for phlorizin

Although the apparent K_m and K_i values for phlorizin differ by an order of magnitude (Table II), the lower value for K_i, determined in the presence of glucose,

* Na^+ entry at the mucosal border of the epithelial cell probably exceeds Na^+ translocation across the cell by a considerable amount. For example, ΔPD values plotted against graded concentrations of glucose or of 6-deoxy-D-glucose yield saturation curves which show an increase in area of 25–35% in the presence of mucosal ouabain (calculated from data in ref. 14). Thus, the mucosal membrane would appear to be quite "leaky" in contrast to the limiting membranes of nerve and muscle.

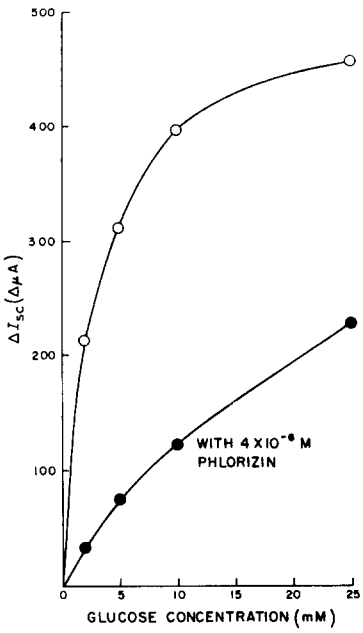


Fig. 6. The influence of graded concentrations of glucose, between 2 and 25 mM, upon ΔI_{sc} values, obtained with paired everted jejunal sacs incubated in KREBS–HENSELEIT bicarbonate buffer, in the absence (upper curve) and in the presence (lower curve) of phlorizin.

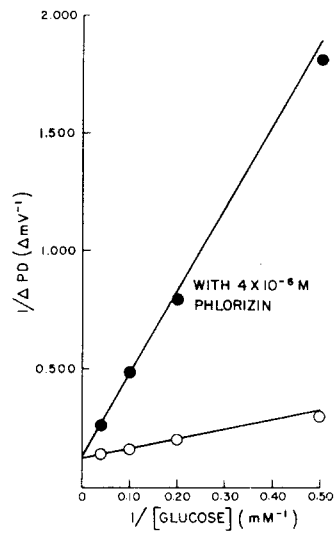


Fig. 7. Double-reciprocal plot of $1/\Delta$PD *vs.* 1/glucose concentration in the absence (lower line) and in the presence (upper line) of phlorizin. Experimental data were obtained with paired everted jejunal sacs incubated in KREBS–HENSELEIT bicarbonate buffer.

suggested the possibility that the phlorizin–carrier interaction may be Na^+-sensitive. This seemed reasonable in view of an apparent mutual allosteric activation between the Na^+–carrier and sugar–carrier interactions[10]. This possibility was tested by a determination of apparent K_m and K_i values for phlorizin in media in which the concentration of Na^+ was fixed at 145, 120 and 24 mequiv. A comparison of these phlorizin values at the three Na^+ concentrations (Table II) clearly indicates the need to consider the possibility of two modes of entry for phlorizin: one, specified by K_i values, is Na^+-sensitive; the other, specified by K_m values, is relatively Na^+-insensitive. The Na^+-sensitive mode may involve interaction of phlorizin with the carrier in a manner directly analogous to that which is characteristic of active sugar transport; the mode which is relatively Na^+-insensitive, on the other hand, may involve a different kind of interaction with the carrier. These possibilities are discussed below.

In the medium containing 145 mequiv Na^+ (Table II), ΔPD and ΔI_{sc} are likely to be predominantly a reflection of the Na^+-sensitive mode of phlorizin interaction with the carrier. The high-affinity characteristic of this interaction would probably result in a rapid saturation of the carrier even at very low concentrations of the glycoside. Under these conditions, phlorizin exit would be favored over its entry, and accumulation, which might be expected by analogy with the actively transported sugars, would not be observed even at higher concentrations of phlorizin.

At 120 mequiv Na^+ (Table II), phlorizin K_m and K_i values tend to approach

each other so that it becomes difficult to distinguish between modes of entry. At 24 mequiv Na^+, phlorizin appears to interact with the carrier preferentially as a substrate. It should be noted that the K_i value at 24 mequiv Na^+ approaches the K_m value at 145 mequiv Na^+ which suggests a Na^+-dependent change in the kind of phlorizin–carrier interaction to one more characteristic of that denoted by the K_m.

The relationship between Na^+-sensitive (K_i) and Na^+-insensitive (K_m) phlorizin–carrier interactions, as well as the low phlorizin concentrations usually employed, may account for the fact that accumulation of this glycoside has not yet been observed. With the present data it is difficult to distinguish between the possibilities of a Na^+-dependent conformational change in the carrier, or of two carriers for the phlorizin interaction, or even an interaction of phlorizin with a macromolecular unit other than that of the carrier. However, evidence in support of an alternative view is presented and discussed below.

Phlorizin–sugar interactions

For two substrates, such as phlorizin and glucose or its various analogs, the particular values for K_i and K_m will depend upon the affinity of each substance for

TABLE III

INFLUENCE OF Na^+ CONCENTRATION ON RELATIONSHIP BETWEEN K_m FOR 1-DEOXY-D-GLUCOSE AND PHLORIZIN K_i VALUES IN RAT JEJUNUM

Na^+ concn. (mequiv.)	*K_m for sugar ($M \times 10^{-3}$)*	*K_i for phlorizin ($M \times 10^{-6}$)*	*K_m/K_i ($\times 10^3$)*
145	7.65	1.02	7.50
120	11.3	1.72	6.58
24	19.5	2.12	9.17
		Mean ± S.D.	7.75 ± 1.04

the carrier site and, although these values may change with varying experimental conditions, the K_m/K_i ratio should remain constant*. The data in Table III indicate that sugar K_m and phlorizin K_i values both increased as the Na^+ concentration in the medium was lowered denoting a strong dependence upon Na^+. The ratio, however, remained essentially unchanged over the range of Na^+ concentrations between 145 and 24 mequiv.

Sugar structure and K_m/K_i ratios

A comparison of K_m/K_i ratios for glucose and a number of its analogs (Table IV) suggests the possibility of using these ratios to assess the effect of a particular structural alteration in the glucose molecule upon the affinity of the altered molecule for

* With the low-affinity sugars, *e.g.*, D-xylose, the effect of phlorizin, particularly at the lower Na^+ concentrations, appears to be one of apparent activation, *i.e.*, ΔPD and ΔI_{sc} values represent phlorizin-dependent *plus* sugar-dependent changes in PD and I_{sc}. This effect is not unexpected since at low Na^+ concentrations the apparent K_m for low-affinity sugars rises sharply and there seems to be a dramatic shift in the nature of the phlorizin–carrier interactions (Table II). However, even in such instances, the absolute value of the K_m/K_i ratio, calculated from the intercepts on the abscissa of Lineweaver–Burk plots (see Fig. 7), remains essentially unchanged.

TABLE IV

RELATIONSHIP BETWEEN SUGAR STRUCTURE AND THE RATIO OF SUGAR K_m TO PHLORIZIN K_i VALUES

Sugar	K_m/K_i *ratio* (*Mean* $\pm$ *S.D.* $\times 10^3$)
D-Glucose	1.43 $\pm$ 0.15
D-Galactose	3.77 $\pm$ 0.78
1,6-Dideoxy-D-Glucose	4.52 $\pm$ 2.14
6-Deoxy-D-Glucose	6.03 $\pm$ 0.40
1-Deoxy-D-Glucose	7.75 $\pm$ 1.04
D-Xylose	16.7 $\pm$ 3.53

the sugar-binding site of the carrier. If it be assumed that the structure of phlorizin permits near maximal interaction with the carrier, then the ratio may be interpreted as follows. As the Na^+ concentration in the medium is decreased, both the apparent K_m for a given sugar and the apparent K_i for phlorizin against that sugar increase proportionately and the ratio remains unchanged. However, the range of K_m values tends to be lower for a sugar having a greater affinity for the carrier while the corresponding range of K_i values tends to be higher since increased concentrations of phlorizin are required to displace a sugar which interacts strongly with the sugar-binding site. In this instance the value of the ratio tends toward a minimum. The converse is true for a lower affinity sugar, *i.e.*, one having a higher apparent K_m value. As a result, glucose, for example, would be expected to yield a lower ratio than xylose, and this has been observed (Table IV). Although the relative affinities of sugars may be determined from their apparent K_m values alone, the K_m/K_i ratio appears to exaggerate differences in affinity, magnifying them in some instances, as with xylose, and minifying them in others, as with glucose. Thus, a comparison of these ratios for two or more structurally related sugars should provide for sharper discrimination in the degree of their interaction with the carrier.

Possible interactions of deoxy analogs of glucose with the carrier

The K_m/K_i values for the deoxy analogs of glucose (Table IV) indicate that interaction with the carrier can occur in the absence of both the C-1 and the C-6 hydroxyl groups (as in 1,6-dideoxy-D-glucose)—a finding which is consistent with the structural requirements as defined by CRANE[17] for sugar active transport. Affinity for the carrier, however, seems to be slightly greater in the presence of the C-1 hydroxyl (as in 6-deoxy-D-glucose) than with the C-6 hydroxyl (as in 1-deoxy-D-glucose). Although the K_m/K_i ratios do not allow a clear distinction in affinity differences among these analogs, the data suggest the possibility for interactions with either or both of these hydroxyl groups. Since the pyran ring is a relatively rigid structure, intramolecular interaction between the hydroxyls does not seem probable. However, if it be assumed that the interacting foci within the sugar-binding site of the carrier remain relatively fixed in a particular conformation, depending upon the Na^+ concentration in the medium, then the affinity differences among the deoxy analogs might be, in part, a reflection of differences in the proportions of eutopic and dystopic interactions[18]. A shift from more eutopic (*e.g.*, 6-deoxyglucose carrier) to more dystopic (*e.g.*, 1-deoxyglucose carrier) interactions would be ex-

pected as a result of steric changes due to the absence of the hydroxyl oxygen atom at C-1 and/or C-6 as well as to the consequent alteration in the balance of polar to non-polar groups. On the other hand, an increase in the apparent K_m, without any significant change in v_{max} has been observed for a number of sugars as the Na^+ concentration in the medium is decreased[10,16]. Such observations are suggestive of Na^+-dependent conformational modifications in the carrier which could contribute to the variations in apparent affinity for the carrier seen with the deoxy sugars.

Possible nature of phlorizin–carrier interactions

The increase in apparent K_m values previously noted for glucose and its actively transported analogs, as the Na^+ concentration of the medium is decreased[10,16] leads to a consideration that the Na^+-dependent K_i values of phlorizin may be the consequence primarily of interactions of the glucose moiety with the sugar-binding site of the carrier. Conversely, the relatively Na^+-insensitive K_m values of phlorizin may depend mainly upon interactions between the carrier and the aglycone moiety of phlorizin. As shown in Table II, reduction of the Na^+ concentration in the medium from 145 to 24 mequiv resulted in more than a 40-fold decrease in the ratio of apparent K_m for phlorizin to its apparent K_i against glucose. This decrease, as implied above, suggests that the interactions characteristic of the aglycone of phlorizin assume relatively greater importance as the interactions of the glycone undergo progressive weakening due to the withdrawal of Na^+ from the medium.

The relative Na^+-independence of apparent K_m values for phlorizin lends support to the view that the cation-binding site of the carrier is in the neighborhood of, but not coincident with, the sugar-binding site. It is possible, however, that Na^+ may interact with one or more of the various functional groups of phlorizin, including the glycone, with a sufficiently low energy of interaction so that the probability would be high for the presence of a Na^+–phlorizin complex. It may be possible to determine experimentally whether or not a complex is formed.

If the above interpretation is correct, one would predict that higher concentrations of phloretin, the aglycone of phlorizin, would be required to saturate the carrier and phloretin would be much less effective as an inhibitor of sugar active transport. These predictions have been confirmed in preliminary experiments. For example, $5 \cdot 10^{-4}$ M phloretin is approximately as inhibitory as $1 \cdot 10^{-6}$ M phlorizin. Similar findings were reported for hamster small intestine *in vitro*[3]. Moreover, phloretin inhibition in rat jejunal sacs appears to affect both the apparent K_m for sugar and v_{max}. Furthermore, carrier–phloretin interactions would be expected to resemble the Na^+-insensitive interactions of phlorizin which, as suggested above, may derive from the aglycone moiety of the latter. Tests of these predictions are in progress.

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