Inhibition of Intestinal Sugar Transport by Phenolphthalein

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SUMMARY

The sodium-dependent intestinal active transport of 3-methyl-D-glucose, a nonmetabolized glucose analog, was competitively inhibited by phenolphthalein ($K_i = 0.7 \times 10^{-4}$ M) *in vitro*. When sugar acccumulation against a concentration difference was prevented by metabolic inhibitors or by replacing Na^+ in the bathing medium with Li^+, phenolphthalein inhibited the specific ion-activated sugar entry process; this is interpreted as direct inhibition of the sugar carrier. Phenolphthalein was extensively bound to tissue protein, and it could not be determined whether it was itself transported across the cell membrane. Phenolphthalein inhibited the sodium-dependent intestinal active transport of α-aminoisobutyric acid as well as its entry in the presence of metabolic inhibitors or in Li^+ medium; this suggests interaction with the amino acid carrier. It is concluded that phenolphthalein affects intestinal sugar transport by competitively inhibiting the sugar carrier. The data do not show to what extent inhibition of the sodium pump (demonstrated by others) may participate in inhibition of sugar transport.

INTRODUCTION

Hand *et al.* (1) reported recently that phenolphthalein (abbreviation: PPT) inhibits glucose transport but not glucose metabolism in the intestine. They suggested that this drug may affect glucose transport in the epithelial cells in the same way as phlorizin, i.e., by specifically inhibiting the glucose entry step. This suggestion gains some support from the observation of Forsling and Widdas (2) that PPT competitively inhibits glucose entry into erythrocytes, a process also inhibited by phlorizin.

In contrast to glucose entry into erythrocytes, however, glucose absorption in the intestine is an active process, dependent on the operation of a sodium pump (3). An inhibitor could affect this process either at the level of the sugar "carrier" proper or at the level of the sodium pump, either directly or by reducing the supply of metabolic energy. The results of Hand *et al.* (1) are consistent with an effect of PPT directly on the sugar carrier, but do not exclude inhibition at the level of the sodium pump; indeed, it was reported recently (4, 5) that PPT and some other cathartic drugs do inhibit active sodium transport in the intestine.

The experiments reported here were designed to describe more fully the mechanism of action of PPT on intestinal sugar transport. The results are discussed in terms of the current carrier theory of membrane transport (6) because it affords a self consistent formal description in agreement with present evidence. It should be understood, however, that this does not necessarily mean acceptance of the phys-

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ical reality of carrier molecules freely mobile within the membrane.

METHODS

The experiments were done *in vitro* on transversely cut segments of small intestine of the golden hamster prepared and incubated according to the procedure of Crane and Mandelstam (7). All incubations were done with gentle shaking at 37° in an atmosphere of 95% O_2–5% CO_2. The incubation medium was Krebs-Henseleit (8) bicarbonate buffer, pH 7.4 or a similar medium in which Na^+ was isosmotically replaced by Li^+ ("lithium medium"). In experiments with high concentrations of sugar the NaCl concentration in the medium was reduced by 50 mM and, to maintain isosmotic conditions, mannitol was added to make the sum of sugar and mannitol concentrations equal to 100 mM. The media also contained a mixture of ^{14}C-labeled and unlabeled 3-*O*-methyl-D-glucose, to give the desired initial concentration. Tracer amounts of ^3H-labeled mannitol were added to measure apparent extracellular space (9). PPT was added, when indicated, as a concentrated ethanolic solution, so that the final ethanol concentration was 1% (v/v); this amount of ethanol had no effect on sugar entry but was nevertheless added to control flasks. Samples of medium or tissue homogenate were deproteinized by the method of Somogyi (10) and their activity was determined by liquid scintillation spectrometry, with a double label counting technique. A liquid scintillation mixture with relatively high efficiency and good water miscibility was used. This modification of Bray's (11) mixture contained 5 g of diphenyloxazole (PPO), 0.5 g of *p*-bis-[2-(5-phenyloxazolyl)]benzene (POPOP), and 80 g of naphthalene per liter of solvent consisting of equal volumes of toluene, *p*-dioxane, and ethyleneglycol monomethyl ether. PPT was determined spectrophotometrically by an adaptation of the method of Huggins and Talalay (12). The sodium and potassium content of the tissue was determined by flame photometry of the deproteinized tissue extract.

3-Methylglucose was a generous gift of Dr. W. L. Glen, Ayerst Research Laboratories. All other chemicals were obtained from commercial sources.

The results are corrected for 80% tissue water content (9) less the apparent extracellular space derived from the mannitol distribution in the same incubation flask.

RESULTS

Figure 1 is a Lineweaver-Burk (13) plot of the effect of PPT on the transport of 3-methylglucose, a sugar which is actively transported by the intestine but is not metabolized (14). The results indicate that inhibition by PPT is competitive. A V_{max} of 2.8 mM/min and a K_m of 18 mM were calculated for 3-methylglucose by extrapolation of these data; this is very close to the apparent K_m of 17 mM calculated by Schultz and Zaluski (15) for the rabbit ileum. The K_i of PPT calculated from this figure is 1.3×10^{-4} M. Nearly identical results were obtained with the closely similar compound, cresolphthalein; its K_i was 1.6×10^{-4} M.

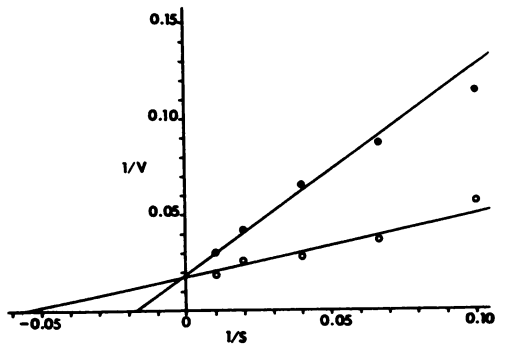

FIG. 1. *Lineweaver-Burk (13) plot of inhibition of 3-O-methyl-D-glucose transport by phenolphthalein.*

Hamster intestinal segments were incubated for 20 min at 37° in Krebs bicarbonate buffer with different sugar concentrations, without phenolphthalein ○——○, or with 0.4 mM (initial concentration) phenolphthalein ●——●. s = mM, v = mmoles/liter of intracellular water/20 min. Each point is the mean of 6 experiments.

The effects of a series of PPT concentrations (50–400 μM) on the transport of

two different concentrations of 3-methylglucose were plotted by the method of Dixon (16). The results confirmed the competitive nature of PPT inhibition and gave a K_i of 1.1×10^{-4} M, which agrees well with the value obtained from the Lineweaver-Burk plot shown in Fig. 1. The above inhibition constants were calculated on the basis of initial concentrations of the inhibitor. However, the PPT concentration in the medium drops appreciably during the incubation (Fig. 2). The constants

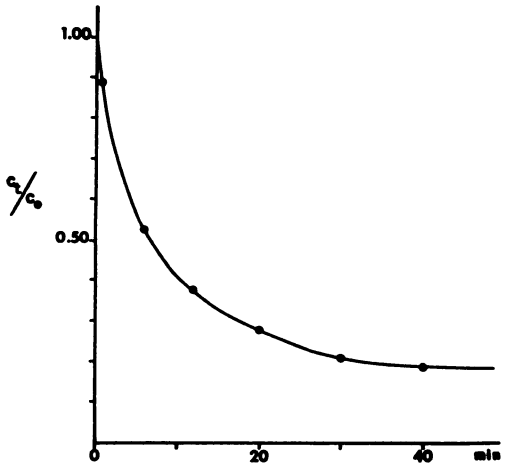

FIG. 2. *Decrease of phenolphthalein concentration in the medium during incubation*

Krebs bicarbonate buffer, 4 ml, containing 0.4 mM phenolphthalein was incubated at 37° with 300 mg hamster intestinal segments. C_0 = initial phenolphthalein concentration, C_t = phenolphthalein concentration after t minutes of incubation.

derived from the Lineweaver-Burk plots were, therefore, recalculated from the geometrical mean of the initial and final concentrations of inhibitor. The corrected values are 0.7×10^{-4} M and 1.0×10^{-4} M for phenolphthalein and cresolphthalein, respectively. The data from the Dixon plot could not be corrected in the same way because the true inhibitor concentrations were not measurable at all concentrations used.

While the competitive nature of the inhibition indicates an action on the presumed carrier, more direct evidence was provided by experiments in which participation of the sodium pump was eliminated. Bihler *et al.* (17) found that sodium activated entry continues, i.e., equilibration of sugar is speeded by Na^+, after active transport is prevented by inhibition of the energy supply to the sodium pump. Table 1 shows that PPT still inhibited sugar entry even when accumulation against a concentration difference was completely abolished by either one of two uncouplers of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenylhydrazone (18) or 4,6-dinitro-*o*-cresol (19). NaF was also added to exclude a possible contribution of energy from glycolysis, which had been suggested by Detheridge *et al.* (20).

As recently demonstrated,[3] lithium, like sodium, activates the sugar carrier but, unlike sodium, does not support active transport, presumably because it is not transported by the sodium pump. In a lithium medium, as in a sodium medium with the sodium pump blocked, the sugar carrier is activated by a specific ion, but the mechanism to maintain an asymmetric distribution of lithium is lacking. The results in Table 2 clearly show that PPT inhibits sugar entry in a lithium medium. These experiments with metabolic inhibitors and with lithium substitution show that the inhibitory effect of PPT may be explained by a direct action on the sugar carrier itself, though an additional, separate inhibition of the sodium pump is not excluded.

If PPT competes with sugar for carrier sites the question arises whether it is itself carried into the cell. Measurements of PPT distribution between tissue and medium showed that it rapidly disappeared from the medium (Fig. 2), but only small amounts of the drug were recovered in the deproteinized tissue extracts. For example, after 20 min of incubation 70% of a PPT dose had disappeared from the medium but less than 2% was found in the deproteinized tissue extract; when the tissue was extracted with ethanol, the recovery of

[3] I. Bihler and Š. Adamič, *Biochim. Biophys. Acta, in press.*

PPT was doubled but still very low. It seems likely that this deficit is due to nonspecific protein binding. This is supported by our observation that when PPT was added to diluted dog plasma, less than 15% were found in the deproteinized supernatant.

TABLE 1

Inhibition of 3-O-methyl-D-glucose transport by metabolic inhibitors and by phenolphthalein

Hamster intestinal segments were preincubated for 20 min at 37° in Krebs bicarbonate buffer with metabolic inhibitors, as indicated, and then incubated for 20 min in fresh buffer with 0.5 mM 3-methylglucose and all the additions listed.

Additions		Percent filling[a]	Percent inhibition
Metabolic inhibitors	Phenolphthalein		
None	0	218[b]	—
DNOC[c] 20 μM + NaF 20 mM	0	79	63
DNOC 20 μM + NaF 20 mM	0.4 mM	31	86
CCP[d] 0.2 μM + NaF 20 mM	0	68	69
CCP 0.2 μM + NaF 20 mM	0.4 mM	32	85

[a] Concentration in intracellular water expressed as percentage of final concentration in medium.
[b] Each figure is the mean of 4 experiments.
[c] 4,6-Dinitro-o-cresol.
[d] Carbonyl cyanide m-chlorophenylhydrazone.

TABLE 2

Inhibition of 3-O methyl-D-glucose entry by phenolphthalein in lithium medium

Hamster intestinal segments were preincubated for 10 min at 37° in Li^+ medium (Krebs bicarbonate buffer with Na^+ replaced by Li^+) and incubated for 40 min in fresh medium with 0.5 mM 3-methylglucose.

	Phenolphthalein, mM			
Parameter	0	0.025	0.1	0.25
Percent filling[a]	51[b]	35	25	14
Percent inhibition	—	31	51	72

[a] Concentration in intracellular water expressed as percentage of concentration in medium.
[b] Each figure is the mean of 4 experiments.

Experiments by others (4, 5) have shown that PPT and similar cathartic drugs inhibit active sodium transport in the intestine. In our experiments it was not possible to evaluate inhibition of the sodium pump in the epithelial cells. However, when compared to control tissue, the intracellular K^+ levels in the whole gut wall were about 10% lower, and the intracellular Na^+ levels correspondingly higher, when 0.4 mM PPT was present during 40 min of incubation. The changes in the epithelial cells may, in fact, be much greater but masked by small changes in the rest of the tissue.

To establish an effect of PPT on the sodium pump which is linked to sugar transport it would be useful to show an effect on a specific process which depends on this same pump. Such a process is the active transport of amino acids which occurs through a separate carrier, but is affected by inhibition of the sodium pump, the function of which seems to be a common requirement for the active transport of several classes of nonelectrolytes (21). Table 3 summarizes data on the transport of α-aminoisobutyric acid, a nonmetabolized amino acid analog. The experiments were performed in the same manner as those on sugar transport except that tissues were deproteinized with 5% trichloroacetic acid. Active transport of the amino acid was inhibited by the same concentrations of PPT which inhibited sugar transport. However, PPT was also inhibitory in the presence of metabolic inhibitors or when

TABLE 3
Inhibition of α-aminoisobutyric acid transport by phenolphthalein

Hamster intestinal segments were preincubated for 20 min at 37° in Krebs of Li^+ medium, and then incubated for 20 min in fresh medium with 0.5 mM α-aminoisobutyric acid.

Medium	Phenolphthalein	Percent filling[a]	Percent inhibition
Krebs	0	155[b]	
	0.4	100	36
Krebs with 0.2 μM CCP[c] and 20 mM NaF	0	100	
	0.4	75	25
Lithium medium	0	34	
	0.4	24	31

[a] Concentration in intracellular water expressed as percentage of the final concentration in the medium.

[b] Each figure is the mean of 3 or 4 experiments.

[c] CCP = carbonyl cyanide *m*-chlorophenylhydrazone in preincubation and incubation medium.

Li^+ medium was used. These data show that the inhibition of amino acid entry by PPT cannot be due only to inhibition of the sodium pump.

DISCUSSION

It is well established that the active transport of glucose in the intestinal epithelial cell is separate from its metabolism; the latter process seems not to be affected by PPT (1). The transport mechanism itself has two components, one mediating the rapid entry of sugar into the cell, the other requiring a sodium pump mediating the supply of energy for accumulation against a concentration difference (17). Of the two hypotheses (3, 21) put forward to explain the Na^+-dependence of intestinal sugar transport, that of Crane and co-workers (3, 22, 23) seems to be better supported by recent evidence (24, 25). It postulates, briefly, that the passage of sugar in either direction across the cell membrane is activated by Na^+ and inhibited by K^+ and that transport against a concentration difference is a consequence of the asymmetrical distribution of ions maintained by the sodium pump. The uptake of amino acids in the intestine and other tissues seems to involve a similar mechanism (26).

In terms of the above concept PPT might inhibit the sugar carrier or the sodium pump or both. Evidence regarding interaction with the carrier will be discussed first. Since it was shown that 6-deoxyglucose entry proceeds at a linear rate for over 20 min (24), and that 1-deoxyglucose reaches a steady state level only after about 40 min (9), it can reasonably be assumed that the results after 20 min incubation with 3-methylglucose also represent a fair approximation of initial sugar entry rates, i.e., the sugar influx. The competitive kinetics of PPT inhibition (Fig. 1 and results of a Dixon plot) should, therefore, be interpreted as indicating an effect on the sugar carrier. Since sugar influx is governed by the extracellular concentrations of ions, these kinetics cannot be a consequence of changes in intracellular ion concentrations resulting from inhibition of the sodium pump. On the other hand, in terms of the model discussed above, intracellular ion levels would influence the steady-state intracellular sugar concentration, reached after long-term incubation (and *in vivo*) and could in that way affect net absorption rates.

A competitive inhibitor could act either by "immobilizing" the carrier or by actually entering the cells in competition with the measured substrate. Our attempts to demonstrate PPT transport failed because PPT is so extensively bound to tissue proteins. The tissue content of PPT reflects primarily this protein binding and cannot be interpreted in terms of distribution in the cellular water space. The uptake of phlorizin by muscle (27) and of its aglucon, phloretin, by erythrocytes (28) follows the same pattern. Alvarado and Crane (29) found that certain phenylglucosides, such as arbutin, were actively transported in the intestine and acted as inhibitors of sugar transport; others, such as phlorizin, were inhibitory, although entry into cells could not be demonstrated. They pointed out that carrier kinetics (6)

predict only very slow net transport of a substrate tightly associated with the carrier [the K_i of phlorizin is around 10^{-6} M (29)]. An alternative explanation, proposed by LeFevre (30), is that inhibitors of the aglucon group, such as phloretin, act not by direct displacement of sugar from carrier sites but by combining with adjacent sites to produce steric interference of sugar access to the carrier. This concept was extended to the phlorizin-like glucosidic inhibitors by Diedrich (31), who suggested that they are attached both to the carrier (via their glucosidic portion) and to adjacent sites (via the aglucon). PPT, which resembles the polyphenolic aglucone inhibitors in structure may also act in this manner, and its transport into the cell would not then be expected. In any case, the strong tissue binding of PPT is sufficient to completely mask any possible contribution of membrane transport to the tissue content; the question of PPT entry into the cell thus remains unresolved.

As a consequence of tissue binding the PPT concentration in the medium drops during incubation, and this must be taken into account when calculating inhibition constants; in this study the geometrical mean of the initial and final concentration was taken as an approximation of the actual concentration of the inhibitor. A correction of this type may also be applicable to the K_i of phlorizin calculated by Alvarado and Crane (29).

The results shown in Table 1, PPT inhibition of sugar entry in the presence of metabolic inhibitors, also indicate that PPT acts directly on the sugar carrier. However, since it is not certain that the sodium pump is completely inhibited in these experiments, it may be argued that sugar entry under these conditions is due to its residual activity; if this is the case, the PPT effect could be explained by inhibition of this mechanism only. This is contradicted, however, by the kinetics of PPT inhibition of sugar entry, discussed above, and by the experiments in Li^+ medium (Table 2). In terms of the proposed mechanism of Li^+-activation of sugar entry, the inhibition in Li^+ medium can be explained by an action of PPT on the sugar carrier, but not by an action on the sodium pump.

If, in addition to its effect on the carrier, PPT inhibits active sugar transport also by affecting the sodium pump, the active transport of amino acids which is dependent on the sodium pump should also be inhibited. Inhibition of active transport of methionine was, indeed, observed (1), but its mechanism was not studied. Active transport of α-aminoisobutyric acid, and its entry in the presence of metabolic inhibitors and in Li^+ medium, were inhibited by PPT to approximately the same degree (Table 3). These results suggest that PPT interferes with the amino acid carrier, and they cannot, therefore, be used as evidence regarding the role of the sodium pump in PPT inhibition. The interaction of PPT with the amino acid carrier is unexpected in view of the fact that phlorizin does not affect it.

Many phenolic, particularly polyphenolic, compounds and their glucosides inhibit sugar transport in a wide variety of tissues (29–31). Active sugar transport in the intestine and the kidney is much more sensitive to glucosides, such as phlorizin. In contrast, erythrocytes and muscles are more sensitive to the aglucons, such as phloretin. PPT appears to belong to the latter group by virtue of its chemical structure (it is a di-(*p*-hydroxyphenyl)-phthalide) and of its greater inhibitory potency in erythrocytes (2) than in the intestine (Fig. 1).

The main conclusion to be drawn from this study is that PPT acts on intestinal sugar transport by competitively inhibiting the sugar carrier. Although the effect of PPT on sodium absorption from the intestinal lumen (4, 5) suggests an inhibition of the sodium pump, our experiments provide no evidence to what degree this may affect sugar transport.

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