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PHENOLPHTHALEIN- AND HARMALINE-INDUCED DISTURBANCES IN THE TRANSPORT FUNCTIONS OF ISOLATED BRUSH BORDER AND BASOLATERAL MEMBRANE VESICLES FROM RAT JEJUNUM AND KIDNEY CORTEX*

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Abstract—Phenolphthalein and harmaline were examined with respect to their effects on the transport functions of purified brush border and basolateral membrane vesicles from rat jejunum and kidney cortex. Phenolphthalein (0.5 mM) inhibited Na^+-coupled D-glucose uptake by intestinal brush border membrane vesicles without affecting Na^+-coupled L-alanine transport, Na^+ transport or Na^+-independent D-glucose transport. In renal brush border membrane vesicles, the same concentration of this drug did not even affect Na^+-coupled D-glucose uptake. At a concentration of 1 mM or higher, however, phenolphthalein rendered both intestinal and renal vesicles leaky to these solutes. In intestinal and renal basolateral membrane vesicles, phenolphthalein at a concentration of 0.5 mM noticeably inhibited (Na^+–K^+)-ATPase activity, but showed no effect on phloretin-sensitive Na^+-independent D-glucose uptake. At 1 mM this drug also inhibited ouabain-insensitive ATPase activity. Harmaline, at concentrations greater than 2 mM, inhibited not only Na^+-coupled D-glucose and L-alanine uptake by both intestinal and renal brush border membrane vesicles, but also Na^+ translocation. The drug, however, affected neither Na^+-independent D-glucose uptake nor the general permeability of these membranes. Harmaline also inhibited (Na^+–K^+)-ATPase activity of intestinal and renal basolateral membrane vesicles without affecting ouabain-insensitive ATPase. It did not influence, however, phloretin-sensitive Na^+-independent D-glucose uptake by these vesicles. These observations suggest that harmaline acts as an inhibitor of Na^+ and Na^+-dependent transport mechanisms in intestinal as well as renal brush border membranes. Phenolphthalein at the lower concentration selectively inhibited certain transport processes in these membranes as well as in basolateral membranes, whereas at the higher concentration it caused widespread structural disturbances, possibly through its chaotropic action on membranes.

Phenolphthalein, a cathartic drug, and harmaline, a plant alkaloid that produces hallucinogenic effects, have been subjects of several recent investigations because of their ability to inhibit various transport activities of mammalian cells. In most of these studies intact intestinal tissue was employed, and it has been reported that these drugs inhibit the uptake of Na^+ and Na^+-coupled D-glucose and neutral amino acids [1–11]. Several studies [12–18] also reported the inhibitory action of the drugs on (Na^+–K^+)-ATPase activity. On the basis of kinetic analyses of these data, it has been postulated that phenolphthalein interacts with carriers of D-glucose and neutral amino acids [7], and that harmaline interacts with Na^+-sites of the nonelectrolyte carrier complexes [10]. The interpretation of these studies, however, is not definitive because of general methodological difficulties inherent in using intact tissue, as well as the existence of a functional polarity in intestinal epithelia [19, 20]. For instance, drug-induced alterations in Na^+-coupled nonelectrolyte transport could be attributed either to direct action of the drugs on intestinal brush border membrane carriers or/and to dissipation of the Na^+-gradient across the brush border membrane. This latter effect could be due to the inhibitory action of the drugs on (Na^+–K^+)-ATPase in the basolateral membrane or on metabolism.

The primary objective of our study was to examine the effects of phenolphthalein and harmaline on the transport functions of intestinal polar epithelial cells by utilizing isolated brush border and basolateral membrane vesicles. Thus, a better understanding of the mechanism of action of these drugs on membrane transport processes, without the complications produced by metabolic effects, could be expected. For this purpose, we developed rapid and simple procedures for isolating functionally active brush border and basolateral membrane vesicles from rat jejunum as well as kidney cortex. The effect of these drugs on transport functions of renal polar epithelia [21] is of physiological significance since these drugs enter the blood circulation.

Vesicular uptake experiments indicated that phenolphthalein (0.5 mM) inhibited the intestinal brush border Na^+-coupled D-glucose uptake, but had

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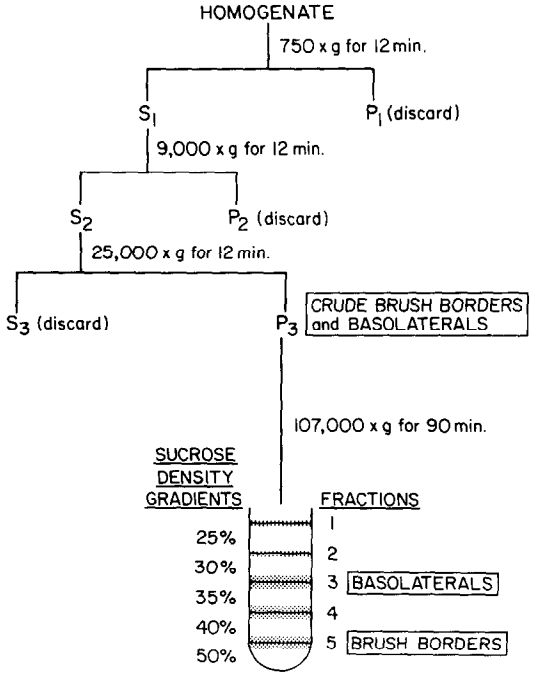
no effect on Na^+-coupled D-glucose transport in brush border membrane vesicles obtained from the proximal tubules of kidney. Low concentrations also inhibited (Na^+–K^+)-ATPase. Concentrations of 1 mM or higher, however, disturbed the general permeability. Harmaline, on the other hand, inhibited various membrane systems having Na^+ binding sites, e.g. Na^+-coupled active nonelectrolyte transport systems, the Na^+ transport system itself, and (Na^+–K^+)-ATPase, with no adverse effect on membrane permeability.

MATERIALS AND METHODS

The jejunal segments and kidneys were removed from six male Wistar rats (weighing approximately 170 g) obtained from the Charles River Research Laboratory, Boston, MA. All the procedures described below were carried out at 4° unless specified otherwise.

Preparation of intestinal brush border and basolateral membrane vesicles. Jejunal segments were everted and vibrated with a Vibro Mixer El (Chemapec Inc., Hoboken, NJ) while suspended in 100 ml of a solution containing 300 mM mannitol and 12 mM Tris–HCl, pH 7.4 (mannitol buffer). Mucus and debris were removed from the everted jejunal surface by vibrating at medium speed for 10 min. After replacing with fresh mannitol buffer, the intestinal segments were vibrated twice at a higher speed, 5/6 of the maximum, for another 10 min each. Practically all of the villous cells were released intact during the last two periods of vibration (monitored by light and electron microscopy). Suspensions containing intact villous cells were combined and filtered through nylon cloth (approximately 189 meshes/in^2). The cells were collected by centrifuging the suspensions at 4500 *g* for 10 min. Then they were resuspended in 30 ml of a solution containing 150 mM mannitol, 10 mM Tris–HCl, 5 mM $MgCl_2$, 30 mM succinate, 0.1 mM $MnCl_2$ and 5 mM potassium phosphate, pH 7.4 (mannitol–succinate buffer).

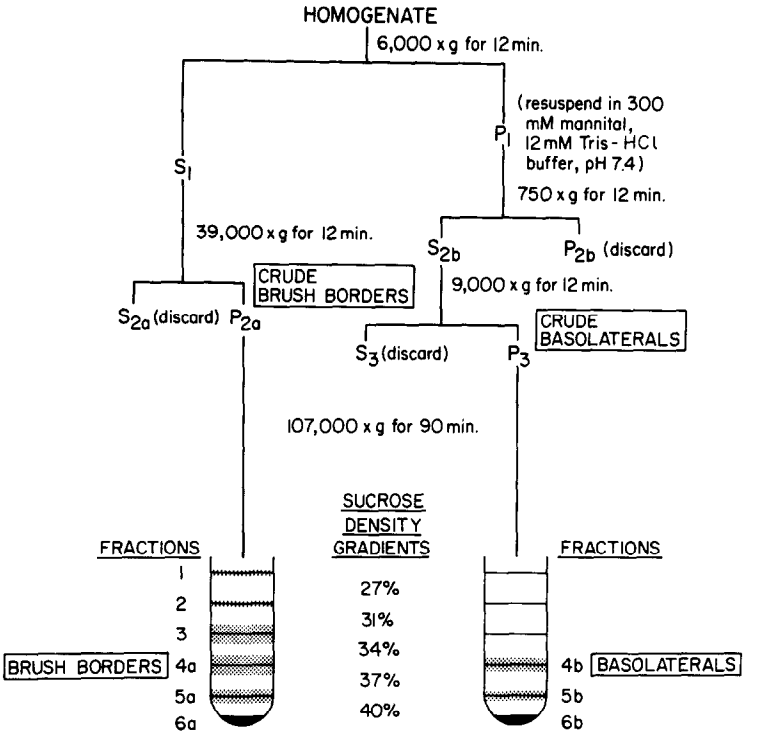


Scheme 1. Isolation of brush border and basolateral membrane vesicles from rat jejunum.

The following procedures were developed for isolation of functionally active brush border and basolateral membranes from the same tissue. The cell suspension was homogenized at 1200 rpm with 75 strokes of a Teflon glass homogenizer (Kontes, clearance 0.0035–0.0045 inches). The homogenate was incubated for 30 min on ice while stirring to allow mitochondria and endoplasmic reticulum to form heavy aggregates, which can be easily separated from plasma membranes by centrifugation [22]. In flow Scheme 1 the differential centrifugation steps are shown in order to obtain fraction P_3, a crude mixture of brush border and basolateral membrane vesicles. P_3 was resuspended in 5 ml of a solution containing 100 mM mannitol, 20 mM Hepes–Tris, 2 mM $MgCl_2$, pH 7.4 (mannitol transport buffer), and homogenized in a 15 ml Dounce homogenizer with a tight fitting pestle (20 strokes).* Then the suspension was layered on a density gradient consisting of 25, 30, 35, 40 and 50% (w/w) sucrose solution containing 2 mM $MgCl_2$ and 2 mM Hepes–Tris, pH 7.4, and centrifuged at 107,000 *g* for 90 min in a SW 50.1 rotor. Each interfacial band was tested for enzyme markers of the brush border membrane, alkaline phosphatase and maltase, and for that of the basolateral membrane, (Na^+–K^+)-ATPase (see Table 1). According to the analysis, most basolateral membranes were located at the interface between 30 and 35% of sucrose (Band 3) and the brush border membranes between 40 and 50% (Band 5). These bands were washed separately with 40 vol. of mannitol transport buffer by centrifugation and usually were resuspended in 2 ml of the same buffer.

Preparation of renal cortical brush border and basolateral membrane vesicles. Twelve decapsulated rat kidneys were used to prepare cortical slices, 1–2 mm thick. The slices, usually weighing around 3 g, were suspended in 30 ml of the mannitol–succinate buffer. This tissue suspension was homogenized three times in a Sorvall Omni-Mixer at the maximum speed for 1 min each. Scheme 2 shows the various steps involved in the isolation of the membrane vesicles. In preliminary experiments we found that the apparent densities of brush border and basolateral membranes were similar. In the presence of Mg^{2+}, however, the majority of the basolateral membranes sedimented in the 6000 *g* pellet fraction containing mainly nuclei and other cellular organelles, while the brush border membrane vesicles stayed in the supernatant fraction. Therefore, as an initial step, the homogenate was incubated for 30 min on ice while stirring in the mannitol–succinate buffer containing Mg^{2+}. Then the homogenate was centrifuged at 6000 *g* for 12 min. The fraction, P_1, containing intracellular organelles and the basolateral membranes, was resuspended in the mannitol buffer without Mg^{2+} or other divalent cations and hom-

* Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.



Scheme 2. Isolation of brush border and basolateral membrane vesicles from rat kidney cortex.

ogenized with 30 strokes of the Teflon glass homogenizer. This step facilitated the separation of the basolateral membranes from intracellular organelles. The crude brush border and basolateral membrane fractions, P_{2a} and P_3, respectively, were suspended in 3 ml of the mannitol transport buffer and were layered on a density gradient consisting of 27, 31, 34, 37 and 40% (w/w) of the sucrose solution containing 2 mM Hepes–Tris and 2 mM $MgCl_2$, pH 7.4. The centrifugation was at 107,000 *g* for 90 min with the SW 50.1 rotor. Analysis of each interfacial band (see Tables 2A and 2B) showed that Band 4a between 34 and 37% of sucrose from P_{2a} retained most of the alkaline phosphatase and maltase activities (the brush border membrane markers), while membrane vesicles of the same density, Band 4, from P_3 showed the highest (Na^+–K^+)-ATPase activity (the basolateral membrane marker).

These bands were washed with 40 vol. of the mannitol transport buffer and usually they were resuspended in 2 ml of the same buffer.

Enzyme assays. The alkaline phosphatase activity of various membrane fractions was estimated according to the method of Hübscher and West [23] by measuring hydrolysis of *p*-nitrophenolphosphate in the presence of potassium fluoride. Maltase activity was measured using the method of Dahlqvist [24]. (Na^+–K^+)-ATPase activity was determined in an incubation medium containing 120 mM NaCl, 7 mM KCl, 5 mM Hepes, 4 mM $MgCl_2$, 0.7 mM EDTA, 0.5 mM deoxycholate, and 4 mM Na_2^+-ATP, pH 7.4, with or without 1 mM ouabain. The inorganic phosphate released was measured by the method of Tsai *et al.* [25]. (Na^+–K^+)-ATPase activity refers to the portion of ATP hydrolysis that was inhibited by ouabain. The remaining activity was referred to as the ouabain-insensitive ATPase activity. It should be noted that harmaline interferes with the colorimetric assay for inorganic phosphate. Therefore, HCl-washed charcoal was used to remove harmaline after termination of the ATPase reaction and before released inorganic phosphate was measured.

The following enzyme activities were assayed in the various membrane fractions to monitor the extent of subcellular organelle contaminations: succinate-cytochrome *c* reductase for mitochondria [26], NADPH-cytochrome *c* reductase for endoplasmic reticulum [27], acid phosphatase using β-glycerophosphate for lysosomes [28], and lactic dehydrogenase for the cytosol [29]. Protein was estimated by the method of Lowry *et al.* [30]. All the assays were performed under the conditions at which reaction rates are linear with respect to reaction time and protein concentrations.

Transport assays. Transport of various solutes into the membrane vesicles was measured using the membrane filtration technique [19, 31]. For measurements of Na^+ or Na^+-coupled nonelectrolyte uptake, the incubation medium contained 100 mM NaSCN, 100 mM mannitol, 2 mM $MgCl_2$, 1% bovine serum albumin (BSA), and 10 mM Hepes–Tris, pH 7.4. Uniformly labeled [^{14}C]-D-glucose or L-alanine was added at a concentration of 150 μM (100 cpm/pmole) when indicated. Carrier-free [^{22}Na]Cl was added when Na^+ uptake was measured. For the Na^+-independent component of the solute uptake, NaSCN was replaced with KSCN. It should be noted that harmaline solubility was greatly reduced in the pres-

Table 1. Specific activities and recovery for marker enzymes of villus cell structures from rat jejunum*

Cell structure	Marker enzyme	Homogenate	Sucrose density gradient fractions					Percentage of enzyme recovery†
			1	2	3	4	5	
Brush border membrane	Alkaline phosphatase	29.9	Not detectable	0.66 (0.8)‡	70.2 (3.0)	145.1 (10.0)	348.2 (40.0)	99.0
	Maltase	1182	430 (0.2)	464 (0.5)	1789 (2.5)	5740 (13.0)	9225 (32.0)	83.0
Basolateral membrane	(Na^+-K^+)-ATPase	95.4	Not detectable	197 (2.5)	1547 (25.0)	180 (4.8)	102 (4.0)	90.0
Mitochondria	Succinate cytochrome *c* reductase	397.4	Not detectable	17.1 (0.06)	27.3 (0.2)	35.5 (0.2)	23.6 (0.2)	85.8
Endoplasmic reticulum	NADPH cytochrome *c* reductase	33.8	93.5 (1.2)	32.5 (1.4)	40.5 (2.7)	34.9 (2.2)	23.8 (1.6)	69.8
Lyosomes	Acid phosphatase	152.0	405 (0.7)	148 (0.8)	142 (1.2)	150 (1.2)	153 (1.3)	96.0
Cytosol	Lactic dehydrogenase	6200	320 (0.02)	220 (0.05)	340 (0.12)	250 (0.09)	178 (0.07)	68.1

* Enzyme activity is expressed as nmoles/mg protein/min.

† Includes recovery at all steps prior to density gradient centrifugation.

‡ Numbers in parentheses represent percentage recovery of the enzyme with respect to the amount present in the homogenate, which is considered to have 100 per cent activity.

Table 2A. Specific activities and recovery of marker enzymes for cell structures from rat kidney cortex (brush border preparation)*

Cell structure	Marker enzyme	Homogenate	Sucrose density gradient fractions						Percentage of enzyme recovery†
			1	2	3	4a	5a	6a	
Brush border membrane	Alkaline phosphatase	335	750 (1.8)‡	725 (4.0)	1333 (10.2)	2268 (19.7)	450 (5.1)	177 (0.7)	85.0
	Maltase	191	200 (0.4)	436 (4.0)	906 (12.0)	1176 (15.0)	327 (7.0)	153 (1.0)	81.0
Basolateral membrane	(Na^+-K^+)-ATPase	131.8	126.2 (0.9)	301.2 (1.6)	274.0 (2.6)	183.4 (1.4)	298.9 (1.8)	340.8 (0.8)	92.8
Mitochondria	Succinate cytochrome *c* reductase	215.0	31.83 (0.01)	8.00 (0.03)	13.71 (0.08)	12.16 (0.06)	5.32 (0.02)	23.50 (0.02)	88.0
Endoplasmic reticulum	NADPH cytochrome *c* reductase	6.07	13.89 (0.21)	Not detectable	2.02 (0.43)	5.58 (0.92)	Not detectable	Not detectable	114.6
Lysosomes	Acid phosphatase	106.7	828.3 (0.7)	378.7 (2.5)	78.0 (1.0)	79.8 (0.7)	97.1 (0.7)	113.0 (0.3)	99.7
Cytosol	Lactic dehydrogenase	1441	54.7 (0.003)	17.4 (0.008)	10.6 (0.009)	35.9 (0.025)	32.4 (0.018)	32.8 (0.005)	93.4

* Enzyme activity is expressed as nmoles/mg protein/min.

† Includes recovery at all steps prior to density gradient centrifugation.

‡ Numbers in parentheses are percentage recovery of the enzyme in the fraction with respect to the amount of enzyme in the homogenate. Enzyme activity in the homogenate is considered to be 100 per cent.

Table 2B. Specific activities and recovery of marker enzymes for cell structures from rat kidney cortex (basolateral membrane preparation)*

Cell structure	Marker enzyme	Homogenate	Sucrose density gradient fractions			Percentage of enzyme recovery†
			4b	5b	6b	
Brush border membrane	Alkaline phosphatase	335	276 (1.7)‡	210 (0.8)	74 (0.6)	85.0
	Maltase	191	101 (1.1)	58 (0.4)	Not detectable	81.0
Basolateral membrane	(Na^+-K^+)-ATPase	131.8	1698.9 (7.5)	1054.2 (6.9)	167.0 (6.8)	92.8
Mitochondria	Succinate cytochrome *c* reductase	215.0	57.05 (0.16)	233.23 (0.75)	19.28 (0.19)	88.0
Endoplasmic reticulum	NADPH cytochrome *c* reductase	6.07	7.02 (0.67)	12.72 (1.44)	2.63 (1.84)	114.6
Lysosomes	Acid phosphatase	106.7	117.5 (0.07)	58.1 (0.4)	30.6 (1.2)	99.7
Cytosol	Lactic dehydrogenase	1441	38.1 (0.015)	39.8 (0.019)	30.2 (0.055)	93.4

* Enzyme activity is expressed as nmoles/mg protein/min.
† Includes recovery at all steps prior to density gradient centrifugation.
‡ Numbers in parentheses are percentage recovery of the enzyme in the fraction with respect to the amount of enzyme in the homogenate. Enzyme activity in the homogenate is considered to be 100 per cent.

ence of SCN ions. Therefore, in experiments with harmaline, NaCl or KCl was used instead of NaSCN or KSCN to prepare the appropriate transport medium. For glucose-dependent Na^+ uptake [32], the incubation medium contained 100 mM KCl, 2 mM $MgCl_2$, 1% BSA, 10 mM Hepes–Tris, pH 7.4, and 100 mM mannitol or a mixture of 50 mM D-glucose and 50 mM mannitol. Then 0.1 mM [^{22}Na]Cl was added finally. Additions of inhibitors or drugs when indicated were made by replacing mannitol iso-osmotically. The incubation media and the membrane suspensions, usually in the mannitol transport buffer containing 1% BSA, were incubated separately at 22°, the reaction temperature, for 5 min. Typically, the transport reaction was initiated by mixing 20 μl of the membrane suspensions (~70 mg protein) with 100 μl of the incubation media. After a designated period of incubation at 22°, the transport activity was stopped by adding 2 ml of ice-cold 150 mM potassium chloride solution containing 0.5 mM phloridzin to brush border membrane vesicles and 0.3 mM phloretin to basolateral vesicles. The suspension was immediately filtered over a Millipore filter (HAWP 0.45 μm pore size) under constant vacuum (27 mmHg). The membrane filters were washed twice with 2 ml of the stopping solution. After air drying, the filters were dissolved in 10 ml of Tritosol scintillation fluid [33]. The radioactivity was counted with a Nuclear Chicago Mark I liquid scintillation system.

In earlier experiments, we found that the intestinal brush border membrane vesicles lost 50 per cent of their Na^+-coupled D-glucose transport activity after 45 min at 22°. When 1% BSA was included in the transport medium, this did not occur during the duration of the experiments (up to 100 min). Therefore, BSA was always included in the transport media as a stabilizing factor.

Data from a typical single experiment are presented throughout this paper. Several similar experiments were performed in each case with variations of less than 10 per cent.

Radioactive D-glucose and L-alanine, uniformly ^{14}C-labeled, were obtained from ICN (Irvine, CA). Carrier-free [$^{22}Na^+$] was purchased from the New England Nuclear Corp. (Boston, MA). Microcrystalline white phenolphthalein, N.F., came from the Monsanto Chemical Co. (St. Louis, MO), and was supplied by the Ex-Lax Pharmaceutical Co., Inc. (Brooklyn, NY). Harmaline, phloridzin and phloretin were obtained from the Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade from standard sources.

RESULTS

Purification of isolated intestinal brush border and basolateral membrane vesicles. Table 1 shows the recoveries and specific activities of the various marker enzymes measured in each of the sucrose gradient bands obtained from P_3 after the density gradient sedimentation (see Scheme 1). Band 5 represented almost 30–40 per cent of the total activities of maltase and alkaline phosphatase in the homogenate. This major brush border membrane fraction showed the highest specific activities of these marker enzymes, being about ten times higher than those of the homogenate. Furthermore, this band showed negligible activities of marker enzymes for mitochondria, endoplasmic reticulum, lysosomes and cytosol. Band 5 had no noticeable enrichment of (Na^+-K^+)-ATPase, the basolateral membrane marker (only 4 per cent of the homogenate). Band 3, on the other hand, contained about 25 per cent of the total (Na^+-K^+)-ATPase activity in the homogenate. The specific activity of this basolateral membrane marker was about fifteen times greater than that of the homogenate. This fraction showed

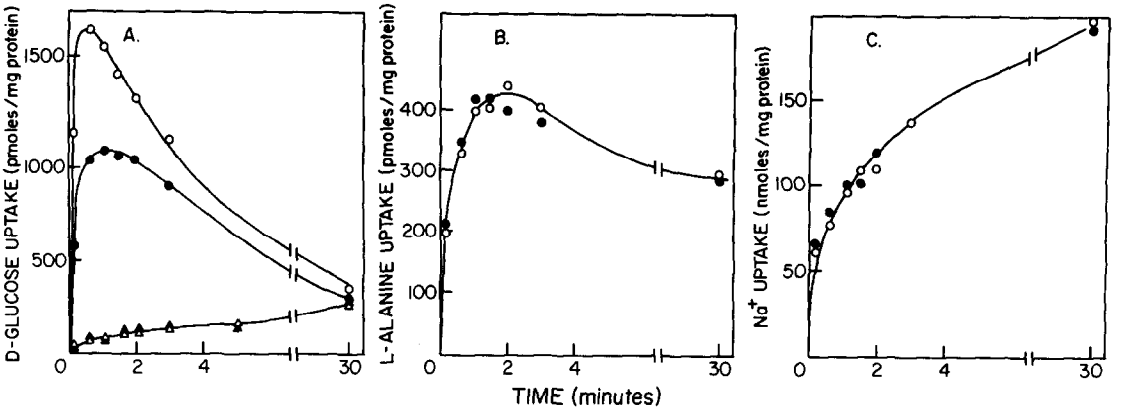


Fig. 1. Time course profiles of D-glucose (A), L-alanine (B) and Na^+ (C) uptake by intestinal brush border membrane vesicles in the absence (open symbols) or in the presence of 0.5 mM phenolphthalein (solid symbols). Panel A: D-glucose uptake in a medium containing either 80 mM NaSCN (circles) or KSCN (triangles). The media were buffered with 20 mM Hepes–Tris, pH 7.4. The concentration of D-glucose was 120 μM, and the incubation was done at 22°. Panels B and C: uptake of L-alanine, 120 μM, or [$^{22}Na^+$] as a tracer in the medium containing 80 mM NaSCN. The other conditions were the same as described for Panel A.

negligible contamination from mitochondria, lysosomes and cytosol. Endoplasmic reticulum and brush border membrane enzymes represent also a minor impurity in Band 3, the total amount of which is less than 3 per cent of the homogenate.

Purification of isolated renal brush border and basolateral membrane vesicles. Tables 2A and 2B show specific activities and per cent recoveries of the various marker enzymes in the sucrose density gradient bands obtained from P_{2a}, the crude brush border membrane fraction, and P_3, the crude basolateral membrane fraction, respectively (see Scheme 2).

As shown in Table 2A, Band 4a was mostly enriched with the brush border membrane markers, maltase and alkaline phosphatase, representing about 15–20 per cent of the total homogenate activities and a 6- to 7-fold purification as compared to the homogenate. No significant contamination from intracellular organelles was detected in this band, which contained only 1.4 per cent of the (Na^+–K^+)-ATPase activity found in the homogenate. As shown in Table 2B, Band 4b was concentrated in (Na^+–K^+)-ATPase, the basolateral membrane marker. The specific activity of this enzyme was about twelve times as high as that of the homogenate. This major basolateral membrane fraction showed no major contamination from mitochondria, endoplasmic reticulum, lysosomes or the cytosol. This fraction also had considerably lower specific activities of the brush border membrane markers as compared with those of the homogenate. Note that a considerable amount of (Na^+–K^+)-ATPase was also located in Band 5b, although the specific activity was lower than that of 4b.

Effect of phenolphthalein on the transport activities of intestinal and renal brush border membrane vesicles. Figure 1A shows the time course profiles of Na^+-coupled and Na^+-independent D-glucose uptake by the intestinal brush border membrane vesicles in the absence and presence of 0.5 mM phenolphthalein. The intestinal membrane vesicles showed typical Na^+-coupled transient uptake of D-glucose in the NaSCN medium. This transient accumulation reached a maximum within 1 min, and decreased gradually to an equilibrium in about 30 min. In the absence of the Na^+ gradient, D-glucose uptake by the membrane vesicles amounted to less than 5 per cent of the Na^+-coupled uptake in the early minutes of the incubation, but reached the same equilibrium level after 30 min. Phenolphthalein at a concentration of 0.5 mM drastically inhibited the transient accumulation of D-glucose, but did not affect the equilibrium level. Also, the drug did not influence Na^+-independent D-glucose uptake by the intestinal brush border membrane vesicles.

Panels B and C of Fig. 1 show the time course profiles of L-alanine and Na^+ uptake by intestinal brush border membrane vesicles in the NaSCN medium in the absence and presence of 0.5 mM phenolphthalein. The drug had no effect on either Na^+-coupled L-alanine or Na^+ uptake.

To examine a dose–response profile of the phenolphthalein action on D-glucose uptake, we measured D-glucose accumulation by the intestinal membrane vesicles in a NaSCN medium during the first 30 sec of the incubation period in the presence of various concentrations of phenolphthalein. At the same time the D-glucose level at equilibrium (30 min) was measured to determine if there were any alterations in the intravesicular volume due to changes in phenolphthalein concentrations. The results are shown in Fig. 2A. As the phenolphthalein concentration was raised to 0.5 mM, the transient accumulation of D-glucose gradually decreased with no noticeable changes in the D-glucose equilibrium level. At phenolphthalein concentrations above 0.6 mM, however, the D-glucose equilibrium level decreased considerably, indicating disturbances in the permeability (structural integrity) of the brush border membranes.

With renal brush border membrane vesicles, however, phenolphthalein at concentrations up to 0.6 mM did not affect Na^+-coupled D-glucose transport.

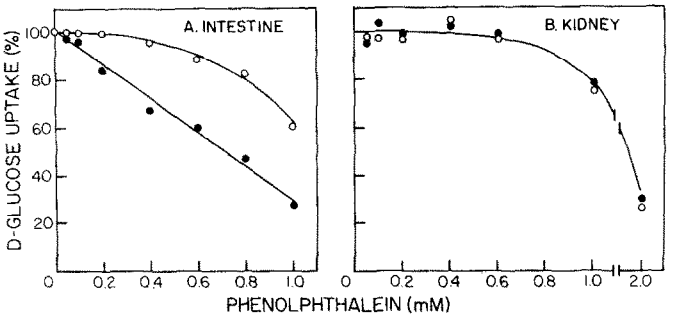


Fig. 2. Dose–response profiles of phenolphthalein action on D-glucose (120 μM) uptake by intestinal (A) and renal (B) brush border membrane vesicles in a medium containing 80 mM NaSCN at 22°. The plots show changes in D-glucose uptake during the first 30 sec of the incubation period (●) or after 30 min of the incubation (○) as a function of phenolphthalein concentration. The other conditions were the same as described in Fig. 1.

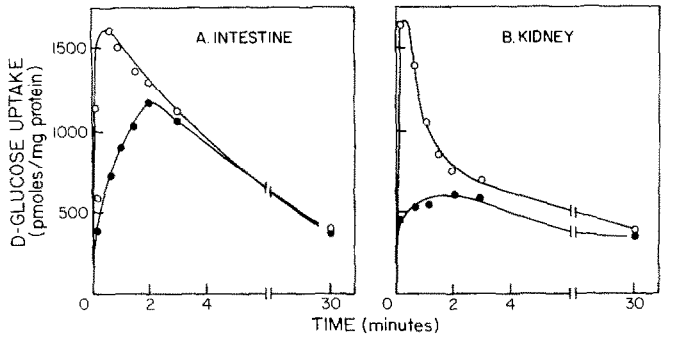


Fig. 3. Time course profiles of Na^+-coupled D-glucose uptake by intestinal (A) and renal (B) brush border membrane vesicles in the absence (○) or in the presence (●) of 10 μM phloridzin. The incubation was in a medium containing 80 mM NaSCN and 120 μM D-glucose at 22°.

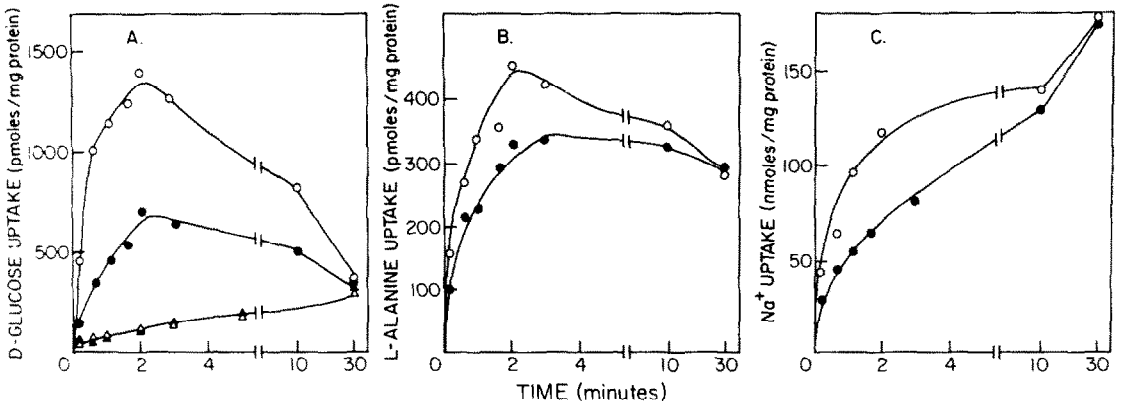


Fig. 4. Time course of D-glucose (A), L-alanine (B) and Na^+ (C) uptake by intestinal brush border membrane vesicles in the absence (open symbols) or in the presence (solid symbols) of 10 mM harmaline. The other conditions were the same as described in Fig. 1.

Also, this drug did not influence Na^+-coupled L-alanine and Na^+ uptake (data not shown). Nevertheless, phenolphthalein at a concentration above 0.6 mM reduced the D-glucose equilibrium level of the renal vesicles close to that of the intestinal brush border membrane vesicles (see Fig. 2B).

To have a better understanding of the action of phenolphthalein on D-glucose uptake, we have examined the effects of phloridzin on Na^+-coupled D-glucose uptake by intestinal and renal brush border membrane vesicles. As shown in Fig. 3, 0.01 mM phloridzin reduced the initial rates of Na^+-coupled D-glucose uptake by intestinal as well as renal brush border membrane vesicles by more than 60 per cent. Phloridzin at a concentration as high as 1 mM did not affect Na^+ and Na^+-coupled L-alanine uptake in

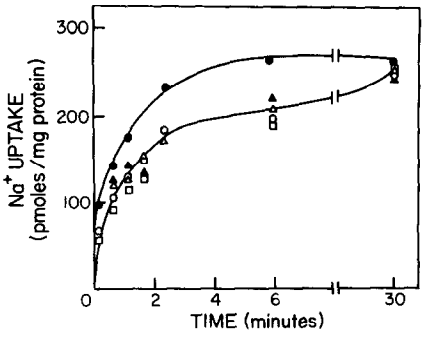


Fig. 5. Time course profiles of uptake of Na^+ at 0.1 mM (NaCl) by intestinal brush border membrane vesicles in a medium containing 80 mM KCl. Key: in the absence of D-glucose (○), in the presence of 50 mM D-glucose (●), and in the presence of D-glucose with 0.5 mM phloridzin (△), with 0.5 mM phenolphthalein (▲) or with 10 mM harmaline (□). See Materials and Methods for details of the procedure.

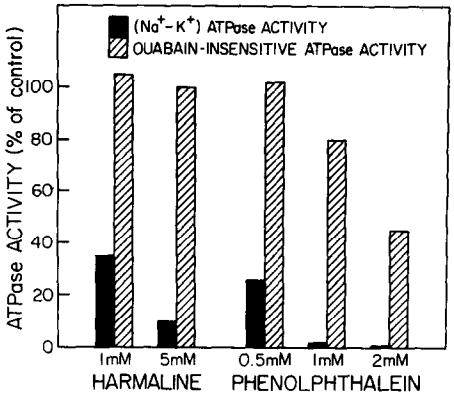


Fig. 7. Effects of harmaline and phenolphthalein on (Na^+–K^+)-ATPase and ouabain-insensitive ATPase activity of intestinal basolateral membrane vesicles. See Materials and Methods for details of the assay conditions.

the intestinal and renal membrane vesicles (data not shown). The primary difference in the action of phenolphthalein from that of phloridzin, therefore, resides in the inability of phenolphthalein to inhibit Na^+-coupled D-glucose uptake by the renal brush border membrane vesicles.

Effects of harmaline on the transport activities of intestinal and renal brush border membrane vesicles. Figure 4 shows the time course profiles for uptake of Na^+-coupled and Na^+-independent D-glucose (A), Na^+-coupled L-alanine (B), and Na^+ (C) by intestinal brush border membrane vesicles in the absence or presence of 10 mM harmaline. Harmaline inhibited the transient accumulation of D-glucose and L-alanine in the NaCl medium and also reduced the initial rate of Na^+ uptake. The drug did not affect, however, the equilibrium levels of these solutes. Furthermore, harmaline did not inhibit Na^+-independent D-glucose uptake by the intestinal brush border membrane vesicles. Similar inhibitory results were obtained with harmaline on the uptake of D-glucose, L-alanine and Na^+ by renal brush border membrane vesicles. The dose–responses of harmaline on the transient accumulation of D-glucose in the NaCl medium (30 sec) were identical in intestinal and in renal brush border membrane vesicles (50 per cent inhibition at 6 mM).

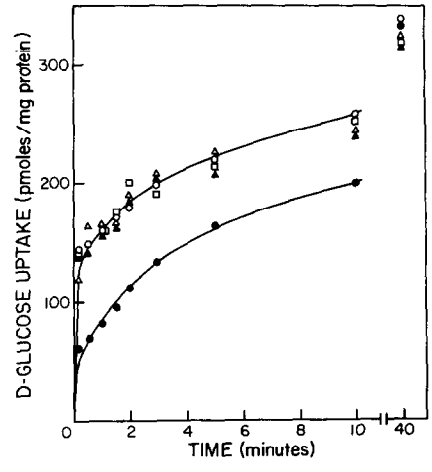


Fig. 6. Time course profiles of D-glucose uptake by intestinal basolateral membrane vesicles in a medium containing 80 mM KCl with no inhibitor (○), 1 mM phloretin (●), 1 mM phloridzin (△), 0.5 mM phenolphthalein (▲) or 5 mM harmaline (□). The other conditions were the same as described in Fig. 1.

Effects of phenolphthalein and harmaline on D-glucose-dependent Na^+ uptake by intestinal brush border membrane vesicles. So far the process of Na^+-coupled nonelectrolyte transport has been examined only with respect to the movement of nonelectrolytes. Rheogenic Na^+ transport is an integral part of this complex transport process. Therefore, we studied Na^+ uptake in response to the movement of D-glucose. Figure 5 shows that 50 mM D-glucose in the incubation medium accelerated the initial rate of Na^+ uptake by the intestinal brush border membrane vesicles from a solution with a Na^+ concentration of 0.1 mM. Phenolphthalein (0.5 mM), harmaline (10 mM) and phloridzin (0.5 mM) abolished the enhanced rate of Na^+ uptake due to D-glucose.

Effects of phenolphthalein and harmaline on the transport functions of intestinal and renal basolateral membrane vesicles. Figure 6 illustrates time course profiles of D-glucose uptake in a KCl medium by intestinal basolateral membrane vesicles in the absence or presence of 0.5 mM phenolphthalein, 5 mM harmaline, 1 mM phloretin or 1 mM phloridzin. Only phloretin inhibited a measurable portion of D-glucose uptake during the early minutes of the incubation period. Final equilibrium levels of D-glucose uptake determined after 40 min were not noticeably altered by the presence of these inhibitors. Similar results were obtained with renal basolateral membrane vesicles (data not shown).

Figure 7 illustrates the effects of harmaline and phenolphthalein on the ATPase activity of intestinal basolateral membrane vesicles. Phenolphthalein at a concentration of 0.5 mM inhibited (Na^+–K^+)-ATPase activity by almost 80 per cent. When the concentration of phenolphthalein was raised to 1 mM, it inhibited not only the residual (Na^+–K^+)-ATPase activity, but it also reduced a considerable

portion of the ouabain-insensitive ATPase activity. Similar results were obtained with renal basolateral membrane vesicles.

Harmaline at a concentration of 1 mM inhibited (Na^+–K^+)-ATPase activity in intestinal membranes by more than 60 per cent, but did not affect the ouabain-insensitive ATPase activity even at 5 mM. In renal basolateral membranes, harmaline at 1 mM also inhibited (Na^+–K^+)-ATPase preferentially, as observed with intestinal membranes.

DISCUSSION

In studies with intact intestine, phenolphthalein at concentrations below 0.5 mM was reported to inhibit Na^+, Na^+-coupled D-glucose and neutral amino acid transport [1–7]. The present study with intestinal brush border membrane vesicles, however, showed that a 0.5 mM concentration of the drug selectively inhibited Na^+-coupled D-glucose uptake without affecting Na^+ or Na^+-coupled L-alanine uptake. We have also shown that phenolphthalein inhibited the rheogenic Na^+ cotransport with D-glucose by the membrane vesicles (see Fig. 5). In intact tissue, however, phenolphthalein, an extremely lipophilic molecule, could rapidly reach the serosal side of intestinal epithelial cells by free diffusion across the brush border, by lateral diffusion along the phospholipid bilayers, and even through the paracellular pathway. Thus, phenolphthalein could inhibit (Na^+–K^+)-ATPase in the basolateral membrane and cause dissipation of a Na^+ gradient across the brush border membrane. The inhibition of Na^+-coupled neutral amino acid uptake observed in intact intestine [1, 6, 7] could, therefore, be a secondary effect of the action of the drug on (Na^+–K^+)-ATPase activity. In any event, the inhibitory effects of phenolphthalein on intestinal Na^+-coupled D-glucose transport and (Na^+–K^+)-ATPase activity could contribute to the laxative action of the drug. Concerning the mechanism of inhibitory action of phenolphthalein on D-glucose uptake, Hand *et al.* [6] pointed out the structural similarity among phenolphthalein, phloridzin and phloretin with the presence of a common phenol group in their structures. In our study, however, we have found a marked difference in the effect of phloridzin and phenolphthalein. For example, phloridzin was very effective in inhibiting Na^+-coupled D-glucose uptake by renal as well as intestinal brush border membrane vesicles, whereas phenolphthalein at a concentration fifty times greater failed to inhibit Na^+-coupled D-glucose uptake by renal brush border membrane vesicles. This differential effect between phenolphthalein and phloridzin suggests that the phenolphthalein inhibition of Na^+-coupled D-glucose uptake by intestinal brush border membrane vesicles should not be attributed to its interaction with the D-glucose binding sites. Phenolphthalein also failed to inhibit phloretin-sensitive D-glucose transport by intestinal basolateral membrane vesicles.

The actions of phenolphthalein could arise from its membrane-perturbing property inherent in its chemical structure, i.e. the two phenolic hydroxyls and three aromatic rings that are essential for laxative activity of various phenolphthalein analogs [34]. Phenolphthalein at 1 mM or higher could possibly cause widespread structural disturbances of biological membranes. At 0.5 mM or less, however, the drug could cause only selective regional disorders of the biological membranes assuming regional variance of the drug affinity. Thus, phenolphthalein could inhibit Na^+-coupled D-glucose transport by intestinal membrane vesicles without affecting Na^+ or Na^+-coupled L-alanine uptake. In addition, the ineffectiveness of phenolphthalein on the renal system may be attributed, on the basis of this hypothesis, to a lower affinity of the drug to the membrane region surrounding the brush border membrane carrier involved in Na^+-coupled D-glucose transport as compared to its counterpart in the intestinal system.

With isolated intestinal brush border vesicles we have shown that harmaline inhibited a variety of functions requiring Na^+ as substrate, i.e. the Na^+-coupled active D-glucose and L-alanine transport systems, the Na^+ transport system in the brush border membrane, and D-glucose stimulated Na^+ transport. These observations support the hypothesis advanced by other investigations [8–10] with intact intestinal tissue that harmaline interacts with Na^+ sites of the nonelectrolyte carrier complex. While our work was in progress, Alvarado *et al.* [35] reported that harmaline inhibited Na^+ and Na^+-coupled D-glucose uptake by isolated brush border membrane vesicles from rabbit jejunum. Our results confirm their finding in another species. Although the studies with intact intestine appear to have correctly predicted harmaline interaction with the Na^+ site of nonelectrolyte carriers, the effectiveness of the drug in inhibiting (Na^+–K^+)-ATPase as shown in the present study certainly suggests that dissipation of the Na^+-gradient across the brush border membrane could also contribute to the inhibitory action of the drug on nonelectrolyte transport.

The difficulty in interpreting transport studies with intact tissue has been more evident with investigations of the effects of harmaline on proximal renal tubular transport processes. Sepúlveda and Robinson [8] reported that harmaline inhibited Na^+-coupled D-glucose and L-phenylalanine uptake by kidney cortical slices. Samarzija *et al.* [11], however, reported that in kidney micropuncture experiments harmaline showed no direct effect on Na^+-coupled D-glucose and amino acid cotransport systems. We have shown with brush border membrane vesicles isolated from the kidney cortex that harmaline inhibited Na^+-coupled D-glucose and L-alanine uptake. Similar observations with rabbit renal cortical brush border membrane vesicles have been reported recently by Aronson *et al.* [36]. In addition, we have shown that harmaline inhibited the translocation of Na^+ alone by the brush border membrane. These observations and the demonstration of a competitive interaction between harmaline and Na^+ transport sites [35, 36] suggest that harmaline probably acts as an inhibitor of Na^+ binding sites. The hallucinogenic effect of harmaline may be attributed to this property of the drug.

The inhibitory effect of harmaline on (Na^+–K^+)-ATPase activity may be more complex. In our study, harmaline inhibited (Na^+–K^+)-ATPase without affecting the ouabain-insensitive ATPase in both

intestinal and renal basolateral membranes. Evidence presented by Robinson [18] indicates that harmaline decreases the apparent affinities for K^+ as well as Na^+ in this enzymatic reaction in brain microsomes. Furthermore, Samarzija *et al.* [11] have reported that harmaline also inhibits renal plasma membrane ATPase activities that are not stimulated by either Na^+ or K^+, i.e. Mg^{2+} and HCO_3^- ATPases. The differences in these findings still remain to be resolved.

In conclusion, the present investigation has demonstrated that pertinent information can be obtained about the mechanism of drug actions on membrane transport functions of polar cells by utilizing relatively pure vesicles of their luminal and contraluminal membranes which have been isolated by rapid and simple procedures.

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