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Examination of the Substrate Stoichiometry of the Intestinal Na^+/Phosphate Cotransporter

Brian E. Peerce
Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

Summary. The substrate stoichiometry of the intestinal Na^+/phosphate cotransporter was examined using two measures of Na^+-dependent phosphate uptake: initial rates of uptake with [^{32}P] phosphate and phosphate-induced membrane depolarization using the potential-sensitive dye $diSC_3(5)$. Isotopic phosphate measures electrogenic and electroneutral Na^+-dependent phosphate uptake, while phosphate-induced membrane depolarization measures electrogenic phosphate uptake. Using these measures of Na-dependent phosphate uptake, three parameters were compared: substrate affinity; phenylglyoxal sensitivity and labeling; and inhibition by mono- and di-fluorophosphates. Na^+/phosphate cotransport was found to have similar Na^+ activations (apparent $K_{0.5}$'s of 28 and 25 mM), apparent K_m's for phosphate (100 and 410 μM), and $K_{0.5}$'s for inhibition by phenylglyoxal (70 and 90 μM) using isotopic phosphate uptake and membrane depolarization, respectively. Only difluorophosphate inhibited Na^+-dependent phosphate uptake below 1 mM at pH 7.4.

Difluorophosphate also protected a 130-kDa polypeptide from FITC-PG labeling in the presence of Na^+ with apparent $K_{0.5}$ for phosphate of 200 μM; similar to the apparent K_m for phosphate uptake, and $K_{0.5}$ for phosphate protection against FITC-PG inhibition of Na^+-dependent phosphate uptake and FITC-PG labeling of the 130-kDa polypeptide. These results indicate that the intestinal Na^+/phosphate cotransporter is electrogenic at pH 7.4, that $H_2PO_4^-$ is the transport-competent species, and that the 130-kDa polypeptide is an excellent candidate for the intestinal Na^+/phosphate cotransporter.

Key Words brush-border membranes · symport · membrane potential · fluorescence

Introduction

Na^+/phosphate cotransport can be divided into two possibilities: electroneutral or electrongenic. While it is generally agreed that two Na^+'s are transported per phosphate on the basis of Na^+ activation of phosphate uptake [1, 3, 4], there is no concensus regarding the valence state of phosphate, which is transported and, therefore, the cotransporter stoichiometry.

The uncertainty with transport-competent phosphate results from the nature of the substrate and its carrier protein. Regardless of the phosphate concentration or pH of the reaction, both monovalent and divalent phosphate will be present in sufficient concentrations to support phosphate uptake in initial rate of uptake experiments. This is certainly true within the constraints on pH imposed by Na^+ gradient-driven phosphate uptake and membrane vesicles. The cotransporter also contributes to the problem. As the medium pH decreases, cotransporter affinity for Na^+ decreases [5]. At a constant Na^+ concentration, phosphate affinity may decrease due to direct effects on the cotransporter rather than substrate availability. Attempts to resolve the question of which valence state of phosphate is transport competent have resulted in monovalent [18], divalent [16], and either [1, 5].

These studies examine the stoichiometry of the intestinal Na^+/phosphate cotransporter comparing substrate affinity and inhibitor sensitivities using Na^+-dependent phosphate-induced membrane depolarization and Na^+-dependent uptake of [^{32}P] phosphate. Monofluorophosphate and difluorophosphate are used as inhibitors of Na^+/phosphate cotransport as has been reported for the phosphate/OH exchanger of mitochondria [8]. The results indicate that the intestinal Na^+/phosphate cotransporter is electrogenic, with a Na^+/phosphate stoichiometry of 2, that monovalent phosphate is the transport-competent species and that if divalent phosphate is transported it is with 5 to 10% of the efficiency of the monovalent form.

Materials and Methods

All chemicals were purchased from Fisher Scientific, Houston, TX, and were reagent grade or better. SDS and all electrophoresis supplies were purchased from Biorad, Richmond, CA. Phos-

phorous pentoxide, ammonium fluoride, nitron, all organic solvents and potassium carbonate were purchased from Aldrich Chemical, Milwaukee, WI. $DiSC_3(5)$ was purchased from Molecular Probes, Eugene, OR. [^{32}P] orthophosphate was purchased from Amersham, Arlington Heights, IL.

Preparation of Brush-Border Membrane Vesicles

Brush-border membrane vesicles were prepared by Ca^{2+} precipitation of intestinal mucosal scrapings as previously described [15, 20]. Following isolation, brush-border membrane vesicles were resuspended in 300 mM mannitol + 10 mM HEPES/Tris, pH 7.5, and stored at liquid N_2 temperature until needed. Storage for up to six weeks had no effect on brush-border enzyme marker activities or Na^+ gradient-dependent uptakes.

The Ca^{2+}-precipitated brush-border membrane vesicles were further purified using 0.02% SDS and a sucrose step gradient centrifugation procedure, as previously described [14]. Briefly, Ca^{2+}-precipitated brush-border membrane vesicles were treated with 0.02% SDS for 15 min in the presence of 100 mM Na^+, 10 mM glucose, 10 mM proline and 50 mM potassium phosphate, pH 7.4, at a final protein concentration of 2 mg/ml. The reaction mixture was layered onto a step gradient of 35% sucrose plus 50 mM potassium phosphate, pH 7.4, overlayed with 10% sucrose plus 50 mM potassium phosphate, pH 7.4, and spun in a fixed-angle rotor for 90 min at 100,000 × *g*. The membrane vesicles were removed from the 10% sucrose/35% sucrose interface by aspiration. The interface fraction was diluted 15-fold with 25 mM HEPES/Tris, pH 7.4, and pelleted at 100,000 × *g* for 60 min. The resultant pellets were resuspended in 25 mM HEPES/Tris, pH 7.4, brought to 150 mM KCl, and centrifuged for 15 min at 10,000 × *g*. The supernatants were collected, diluted fivefold with 25 mM HEPES/Tris, pH 7.4, and pelleted at 100,000 × *g* for 60 min. The pellets were collected, resuspended in 25 mM HEPES/Tris, pH 7.4, and centrifuged at 100,000 × *g* for 60 min. This step was repeated twice. The pellets from the final wash were resuspended in 300 mM mannitol plus 10 mM HEPES/Tris, pH 7.4, and stored at liquid N_2 temperature until needed.

The SDS-BBM vesicles were enriched in the brush-border enzyme markers alkaline phosphate, and γ-glutamyl transpeptidase three to fivefold as compared to Ca^{2+}-precipitated brush-border membrane vesicles. Na^+-dependent glucose uptake, Na^+-dependent phlorizin binding and Na^+-dependent phosphate uptake are fivefold to sevenfold enriched. Na^+/proline cotransport is fourfold enriched relative to the Ca^{2+}-precipitated brush-border membrane vesicles.

Na^+-Dependent Phosphate Uptake

Na^+-dependent phosphate uptake was performed using the initial rate of uptake measurements and a rapid mixing/rapid filtering technique [20]. Na^+-dependent uptake is defined as uptake in the presence of 100 mM *cis* NaCl minus uptake in the presence of 100 mM *cis* KCl, 25 mM HEPES/Tris, pH 7.4, using 3-sec uptakes of 100 μM [^{32}P] orthophosphate at 22°C. Osmolality was maintained with mannitol.

In experiments examining the effects of Na^+ concentrations on phosphate uptake, the Na^+ concentrations were varied from 5 to 150 mM. Osmolality was maintained at 300 mOsm with mannitol.

Synthesis

FITC-PG

Fluorescein isothiocyanate-phenyl glyoxal was synthesized as previously described [14]. Purity of FITC-PG was determined using TLC on silica gel in 70% phenol; 30% dichloromethane following a 10-min reaction with 10 mM arginine. FITC-PG was analyzed by IR spectrophotometry as previously described [14].

Fluorophosphates

Monofluorophosphate and difluorophosphate were synthesized by established methods [11]. Following isolation of each derivative, it was run on a 30 × 1.5 cm silica gel column in methanol : NH_4OH : 10%TCA : H_2O (50 : 15 : 5 : 30). Absorbance of the eluants was monitored at 240 nm. To ensure that no monofluorophosphate contaminated the difluorophosphate, prior to addition of potassium nitrate to form the potassium salt, ammonium difluorophosphate was recrystalized three times from ethanol. Ammonium monofluorophosphate is sparingly soluble in ethanol [6].

SDS-PAGE

SDS polyacrylamide gel electrophoresis was performed on 7.5 or 10% slab gels according to the method of Laemmli [10]. The gels were stained with coomassie blue. Parallel tracks loaded with 200 μg of SDS-BBMV protein were examined for FITC-PG labeling in the presence and absence of substrates, or 100 mM NaCl and varying amounts of monofluorophosphate or difluorophosphate. FITC-PG was read at 490 nm using a Gilford 260 UV/VIS spectrophotometer and gel-scanning attachment.

Membrane Potential Measurements

Na^+/phosphate cotransporter membrane depolarization was examined on an SLM SPF 500c spectrofluorometer using the membrane potential-sensitive dye $diSC_3(5)$. SDS-BBMV protein was loaded with 100 mM potassium gluconate, 50 mM TMA gluconate, 20 mM potassium borate buffer, pH 7.4, by overnight incubation at 4°C. Protein (100 μg) was added to 100 mM sodium gluconate or 100 mM potassium gluconate, 50 mM TMA gluconate, 20 mM potassium borate, pH 7.4, and 10 μM $diSC_3(5)$. Dye fluorescence was monitored continuously using an excitation wavelength of 622 nm and an emission wavelength of 655 nm. Slit widths were 2 nm. The increase in dye fluorescence upon addition of potassium phosphate was recorded. The effect of mono- and di-fluorophosphate was examined by comparison to 1 mM potassium phosphate. All measurements were performed at 22°C with the spectrofluorometer set in the ratio mode. The reference cuvette contained the appropriate solution composition and membrane vesicles, without dye. In these experiments, the enhancement of $diSC_3(5)$ fluorescence by phosphate was recorded as a function of time, and the signal, 20 sec after the addition of phosphate, taken as the new fluorescence intensity in the determination of ΔF.

Fluorescence Quenching Experiments

SDS-BBM vesicles were labeled with FITC-PG following pretreatment with 200 μM phenylglyoxal in the presence of 100 mM Na^+, 10 mM phosphate and 50 mM potassium borate, pH 7.5. Following a 30-min incubation at 22°C, the reaction media was diluted 20-fold with ice-cold buffer and centrifuged at 100,000 × g for 60 min. The pellets were resuspended in 300 mM mannitol plus 10 mM HEPES/Tris, pH 7.5, and treated with 50 μM FITC-PG in the presence and absence of 100 mM NaCl and 10 mM phosphate in 50 mM potassium borate, pH 7.5, for 30 min in the dark at room temperature. The reaction was stopped as described above. Unreacted FITC-PG and substrates were removed by centrifugation, as described above. The pellets were resuspended in 300 mM mannitol plus 10 mM HEPES/Tris, pH 7.5. In experiments examining protection by mono- and di-fluorophosphate, the fluorophosphate was substituted for 10 mM phosphate in the presence of 100 mM Na^+ during exposure to FITC-PG.

Quenching of FITC-PG fluorescence by monovalent cations was performed on an SLM SPF-500c spectrofluorometer at 22°C in the ratio mode. The experiments were performed in 50 mM potassium borate buffer, pH 7.4, using 100 μg of FITC-PG labeled protein. FITC-PG was excited at 492 nm and emission was recorded at 522 nm. Slit widths were set at 2 nm. Membranes labeled with FITC-PG in the presence of substrates were used in the reference cuvette. All fluorescence data are reported as corrected emission spectra.

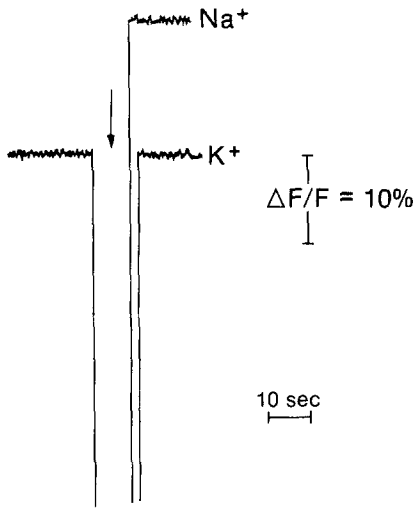

Fig. 1. The effect of phosphate on $diSC_3(5)$ fluorescence. SDS-BBM vesicles were incubated overnight as described in Materials and Methods. SDS-BBMV protein (200 μg) was added to the cuvette in the presence of 100 mM Na^+ or 100 mM K^+, 100 mM TMA^+, 25 mM potassium borate, pH 7.4, and 10 μM $diSC_3(5)$ and the fluorescence recorded. At the arrow, 1 mM potassium phosphate, pH 7.4, was added. The results shown are from a single experiment and are representative of 12 separate determinations

Results

Substrate Affinity: Na^+-Activation of Phosphate Uptake

Na^+ stimulation of phosphate uptake into the SDS-BBM vesicles was examined using two types of measurements. [^{32}P] phosphate uptake was examined under Na^+ or K^+ gradient conditions at pH 7.4, or phosphate uptake was measured using the potential-sensitive dye $diSC_3(5)$. The carbocyanine dyes have been used in renal and intestinal brush-border membrane vesicles to monitor Na^+-dependent cotransporter induced membrane depolarization upon addition of organic substrate [4, 17, 21].

Figure 1 shows a membrane depolarization experiment using the carbocyanine dye and 100 μg of SDS-BBMV protein. In the presence of 100 mM *cis*, Na^+ dye fluorescence increases upon addition of 1 mM phosphate to 15% above the initial fluorescence. In the presence of K^+, phosphate has no effect. The increase in dye fluorescence was stable up to 1 min after the addition of phosphate under these experimental conditions.

The Na^+ activation of phosphate uptake into SDS-BBM vesicles is summarized in Table 1. Na^+ activation of [^{32}P] phosphate uptake displayed a sigmoidal relationship with a $K_{0.5}$ of 28 ± 3 mM (n = 6). Hill plots of this data yield a Hill coefficient of 1.9 ± 0.2 (n = 6) indicating that at least 2 Na^+'s are transported per phosphate. Membrane depolarization was also a sigmoidal function of the Na^+ concentration with an apparent $K_{0.5}$ of 25 ± 3 mM (n = 4). The Hill coefficient was 1.6 ± 0.2 (n = 4).

Table 1. Substrate affinity[a]

Substrate	Na^+-dependent phosphate uptake			
	[^{32}P] Phosphate		Membrane depolarization	
	K_m (mM)	n	K_m (mM)	n
Na^+	28 ± 3	1.9 ± 0.2	25 ± 3	1.6 ± 0.2
Phosphate	0.1 ± 0.015		0.41 ± 0.03	

[a] Apparent substrate affinities were determined from Woolf-Augustinin-Hofstee plots using initial rates of labeled phosphate uptake as described in Materials and Methods or membrane depolarization as measured by carbocyanine dye fluorescence. For initial rate of uptake experiments, a diffusional component was determined and subtracted from the uptake measured in the presence of Na^+ as previously described [9]. The results shown for Na^+ activation of labeled phosphate uptake were determined from triplicate uptakes and are the means ± SEM of six separate determinations. The results shown for substrate-induced membrane depolarization were determined from duplicate measurements and three separate determinations. The results shown for the effect of phosphate concentration on Na^+-dependent phosphate uptake were determined from triplicate measurements and are the means ± SEM of four separate determinations.

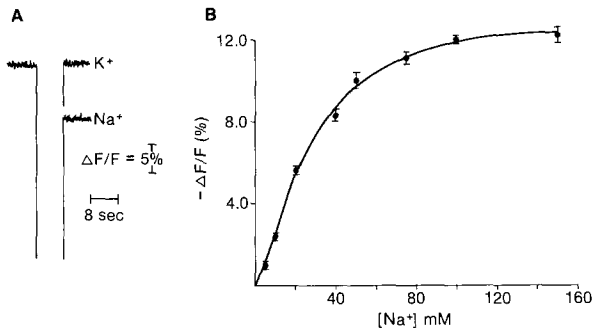

Fig. 2. Quenching of FITC-PG fluorescence by Na^+. SDS-BBMV membranes were pretreated with phenylglyoxal in the presence of substrates followed by FITC-PG as described in Materials and Methods. Fluorescence of 100 μg of treated membranes was recorded in 2.5 ml of 50 mM Tris-Borate + 2 mM EDTA, pH 7.4, at 522 nm with excitation at 492 nm. Slit widths were 2 nm. (*A*) Tracing of FITC-PG fluorescence in the absence of salt, and following addition of 50 mM KCl or 50 mM NaCl. (*B*) Dose-response curve of the Na^+-induced quenching of FITC-PG fluorescence. The points are means ± SD of eight determinations on four different membrane preparations

Phosphate Affinity

The apparent K_m for phosphate uptake at pH 7.4 and 100 mM Na^+ was examined by [^{32}P] phosphate uptake. Table 1 summarizes the effect of phosphate concentration on phosphate uptake. The apparent K_m for phosphate was 100 ± 15 μM (n = 4). A similar series of experiments using membrane depolarization as a function of phosphate concentration indicated an apparent K_m for phosphate of 410 ± 30 μM (n = 3). The higher K_m for membrane depolarization is consistent with other studies of amino acid [19] and organic acid [21] uptake as measured by membrane depolarization, with membrane potential-sensitive phosphate uptake, and inconsistent with electroneutral Na^+/phosphate cotransport.

Na^+-Induced Conformational Change

Several laboratories have reported that Na^+ increases the apparent affinity of the cotransporter for phosphate [2, 5], and that phenylglyoxal derivatives inhibit intestinal [12, 13] and renal [2] Na^+/phosphate cotransport. Fluorescent group specific reagents have been used with a variety of membrane transport proteins to examine substrate-induced changes in protein conformation including the intestinal Na^+/glucose [15], Na^+/proline [22], and renal Na^+/glucose [23] cotransporters. A fluorescent phenylglyoxal derivative has been reported to label the intestinal Na^+/phosphate cotransporter in a substrate-sensitive manner [12, 14]. The effect of monovalent cations on SDS-BBM vesicles labeled with FITC-PG at or near the cotransporter phosphate site is shown in Fig. 2.

Figure 2*A* shows the fluorescence emission of FITC-PG labeled SDS-BBM vesicles in 50 mM potassium borate + 2 mM EDTA, pH 7.4. Addition of 50 mM Na^+ results in a 13% quenching of FITC-PG fluorescence. Addition of 50 mM K^+ did not affect the fluorescence. Rb^+, Cs^+, NH_4^+ or choline also had no effect on FITC-PG fluorescence emission. A small quenching was observed upon addition of 100 mM Li^+. The fluorescence quenching is shown as a function of Na^+ concentration in Fig. 2*B*. Quenching is saturable function of the Na^+ concentration with an apparent $K_{0.5}$ for Na^+ of 25 ± 5 mM (n = 6). The sigmoidal relationship between the Na^+ concentration and the fluorescence quenching of FITC-PG emission is similar to that seen for Na^+ activation of phosphate uptake. The results when analyzed by a Hill plot yield a Hill coefficient of 1.55 ± 0.2 (n = 6). These results are consistent with a Na^+-induced conformation change in the intestinal Na^+/phosphate cotransporter at or near the cotransporter phosphate site resulting from the cooperative binding of 2 Na^+'s.

Inhibitors of Na^+-Dependent Phosphate Uptake: Monofluorophosphate and Difluorophosphate Inhibition of Na^+/Phosphate Cotransport

Mono- and di-fluorophosphate were examined for their effects on Na^+-dependent phosphate uptake using [^{32}P] phosphate and $diSC_3(5)$ fluorescence. The results of these experiments are presented in Fig. 3 and summarized in Table 2. Using either Na^+-dependent uptake of labeled phosphate or membrane depolarization as the measure of cotransporter activity, only difluorophosphate significantly affects phosphate uptake at concentrations below 1 mM. At 5 mM, monofluorophosphate

Table 2. Effect of monofluorophosphate and difluorophosphate on Na^+-dependent phosphate uptake

Inhibitor	Na^+-dependent phosphate uptake			
	[^{32}P] Phosphate		Membrane depolarization	
	$\%I_{max}$	$K_{0.5}$	$\%I_{max}$	$K_{0.5}$
Monofluorophosphate	20	>5 mM (n = 3)	25	>5 mM (n = 5)
Difluorophosphate	90	160 ± 22 μM (n = 4)	100	210 ± 20 μM (n = 5)

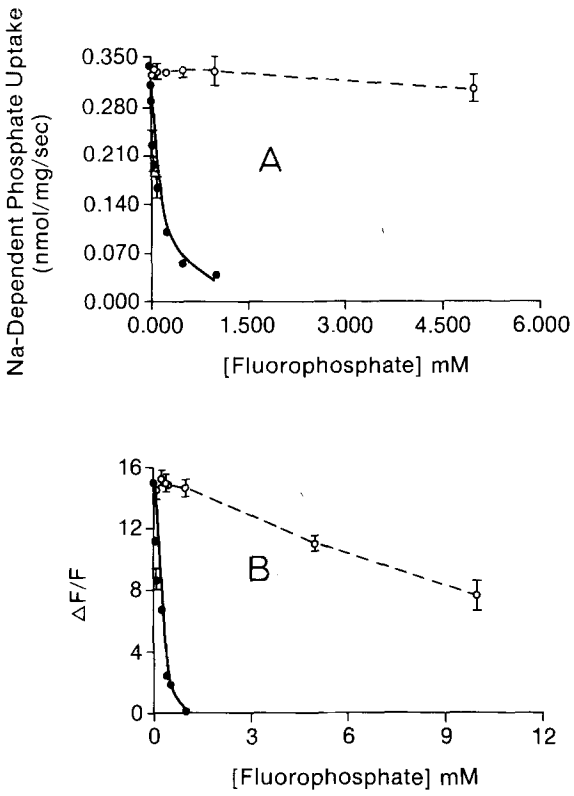

Fig. 3. Effect of fluorophosphate on Na^+-dependent phosphate uptake. Na^+-dependent [^{32}P] phosphate uptake and Na^+-dependent phosphate-induced membrane depolarization were determined as described in Materials and Methods. (*A*) Effect of fluorophosphate on Na^+-dependent [^{32}P] phosphate uptake. SDS-BBMV (50 μg) in 300 mM mannitol + 25 mM HEPES/Tris, pH 7.4, were added to uptake buffer as described in Materials and Methods. Results are averages ± SD of triplicate determinations and representative of four separate experiments. (*B*) Effect of fluorophosphate on Na^+-dependent membrane depolarization. Results are averages ± SD of duplicate determinations and representative of three separate experiments

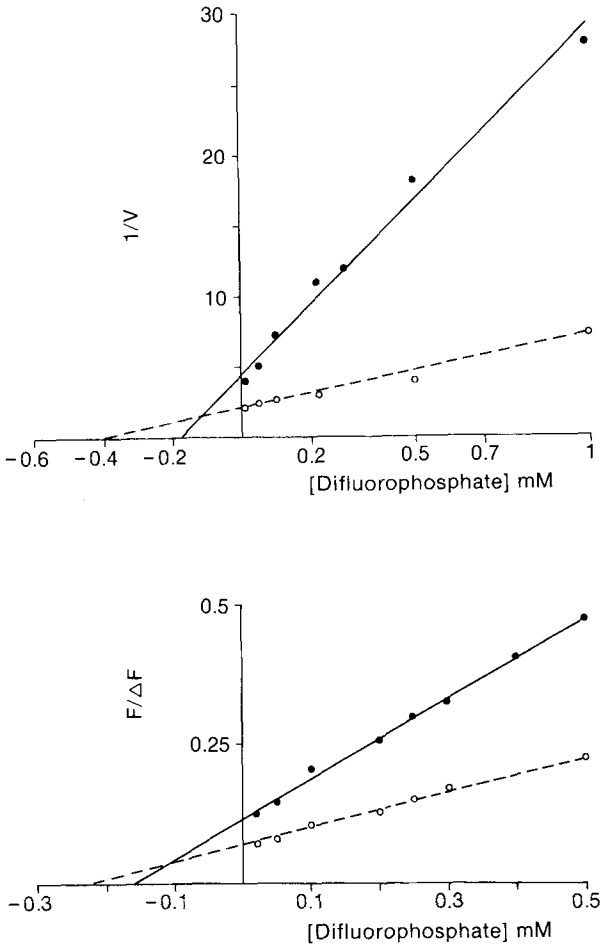

Fig. 4. Dixon plot of the effect of difluorophosphate on Na^+-dependent [^{32}P] phosphate uptake. Na^+-dependent phosphate uptake was determined as described in Materials and Methods. Results are means ± SD of triplicate determinations and three separate experiments. Solid circles, solid line: [phosphate] = 0.1 mM; open circles, dashed line: [phosphate] = 0.5 mM

inhibition of Na^+/phosphate cotransport was less than 30%. In contrast, difluorophosphate inhibited 50% at 200 μM.

Inhibition by difluorophosphate at pH 7.4 was examined using Dixon plots at two phosphate concentrations (closed circles, solid line 100 μM and open circles, dashed line 0.5 mM). The results are shown in Fig. 4. The plots indicate that difluorophosphate is a competitive inhibitor with respect to phosphate using either measure of Na^+-dependent phosphate uptake. The inhibition lines meet above the *x*-axis at 130 μM difluorophosphate for membrane depolarization and 160 μM difluorophosphate using [^{32}P] uptake. The good agreement in the apparent K_I for difluorophosphate using these two measures of Na-dependent phosphate uptake is consistent with one mode of Na/phosphate cotransport, and that mode being electrogenic.

Phenylglyoxal Inhibition of Na^+-Dependent Phosphate Uptake

Phenylglyoxal sensitivity and substrate protection was examined using [^{32}P] phosphate uptake and membrane depolarization. Phenylglyoxal inhibition of phosphate uptake as measured by isotopic phosphate uptake and brush-border membrane depolarization is shown in Fig. 5. Inhibition of $diSC_3(5)$ fluorescence enhancement by phenylglyoxal (Fig. 5*A*) is half-maximal at 90 ± 10 μM (n = 3) similar to the $K_{0.5}$ for phenylglyoxal inhibition of Na^+-dependent phosphate uptake using [^{32}P] phosphate (70 ± 10 μM, n = 4) (Fig. 5*B*). The maximum inhibition observed is 90 ± 5%. This is also similar to the inhibition observed using isotopic phosphate uptake [2, 13, 14].

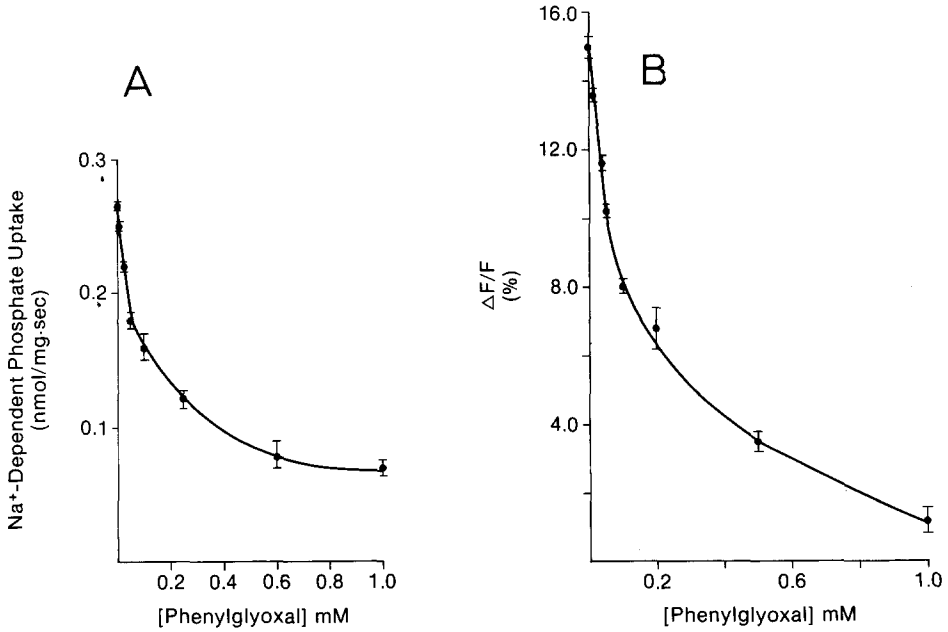

Fig. 5. Effect of phenylglyoxal on Na^+-dependent phosphate uptake. (*A*) Effect of phenylglyoxal on Na^+-dependent [^{32}P] phosphate uptake. SDS-BBMV (500 μg) were treated with varying concentrations of phenylglyoxal for 30 min at 22°C in 50 mM potassium borate, pH 7.4, and 300 mM mannitol. The unreacted phenylglyoxal was removed by centrifugation and the pellets resuspended in 300 mM mannitol, 25 mM potassium borate, pH 7.4. Na^+-dependent uptake was determined as described in Materials and Methods. Results are means ± SD of triplicate determinations and representative of four separate experiments. (*B*) Effect of phenylglyoxal on Na^+/phosphate-induced membrane depolarization. SDS-BBMV were treated with phenylglyoxal as described above, except 150 mM potassium gluconate was substituted for mannitol during the phenylglyoxal incubation and stop solutions. Membrane depolarization was measured by the enhancement of $diSC_3(5)$ fluorescence as described in Materials and Methods. Results are means ± SD of duplicate determinations and representative of three separate experiments

Identification of the Na^+-Phosphate Cotransporter

A fluorescent derivative of phenylglyoxal and SDS-BBM vesicles have been used to identify the Na^+/phosphate cotransporter as a 130-kDa polypeptide band on SDS-PAGE [12, 14]. This polypeptide is labeled with FITC-PG in the absence of substrates suggesting that FITC-PG binds at or near the cotransporter phosphate site. Difluorophosphate and monofluorophosphate were used as phosphate analogues to protect the cotransporter from FITC-PG labeling in the presence of 100 mM Na^+. SDS-PAGE was performed on 7.5% reducing gel as previously described [14, 15]. Individual tracks were scanned at 490 nm for fluorescein absorbance. The results are presented in Figs. 6 and 7.

The presence of Na^+ and phosphate results in the selective protection of a 130-kDa polypeptide from FITC-PG labeling [14]. Addition of 100 mM Na^+ and 1 mM phosphate results in a 70 ± 8% (n = 3) decrease in FITC-PG binding to a 130-kDa polypeptide (*data not shown*). Figure 6 shows a similar experiment substituting 1 mM monofluorophosphate (*B*), or 1 mM difluorophosphate (*A*). Monofluorophosphate has little effect (5 ± 1%, n = 3) on FITC-PG labeling. In contrast 1 mM difluorophosphate inhibits FITC-PG binding as effectively as 1 mM phosphate (80 ± 4%, n = 3). The absence of FITC-PG labeling agrees with the effect of difluorophosphate on Na^+-dependent uptake as measured by radiolabeled [^{32}P] phosphate uptake or $diSC_3(5)$ fluorescence enhancement. Difluorophosphate protection against FITC-PG labeling of the 130-kDa polypeptide is shown in Fig. 7 as a function of difluorophosphate concentration. The apparent $K_{0.5}$ for protection in the presence of 100 mM Na^+ is 0.23 ± 0.02 mM (n = 3) in good agreement with the apparent K_I of 200 μM seen for inhibition of the Na^+-dependent phosphate uptake (Table 2). These results suggest that the 130-kDa polypeptide identified as a candidate for the intestinal Na^+/phosphate cotransporter is an electrogenic Na^+/phosphate cotransporter.

Discussion

The intestinal Na^+/phosphate cotransporter substrate stoichiometry was examined using two mea-

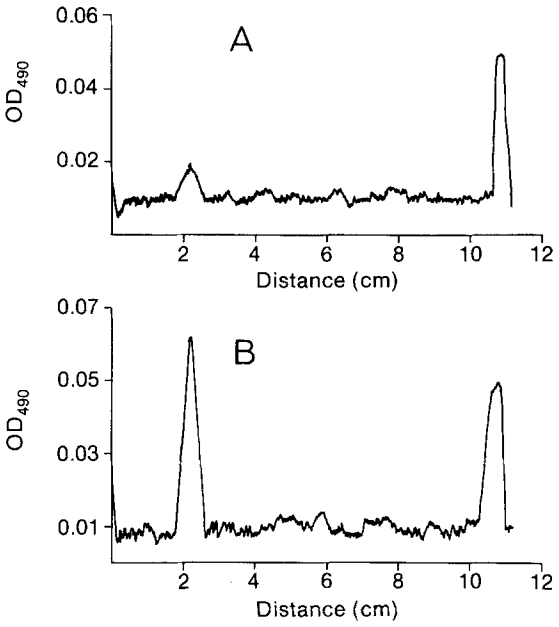

Fig. 6. SDS polyacrylamide gel electrophoresis of FITC-PG-labeled SDS-BBMV. FITC-PG labeled SDS-BBMV were pretreated with 0.2 mM phenylglyoxal in the presence of substrates as described in Materials and Methods. Pretreated vesicles were subsequently treated with 50 μM FITC-PG in the presence of (*A*) 100 mM Na^+ and 1 mM difluorophosphate, or (*B*) 1 mM monofluorophosphate. SDS-PAGE was performed on 7.5% reducing slab gel by the method of Laemmli [10]. Following electrophoresis, individual sample tracks were scanned on a Gilford spectrophotometer at 490 nm as described in Materials and Methods. Results are representative of three separate experiments

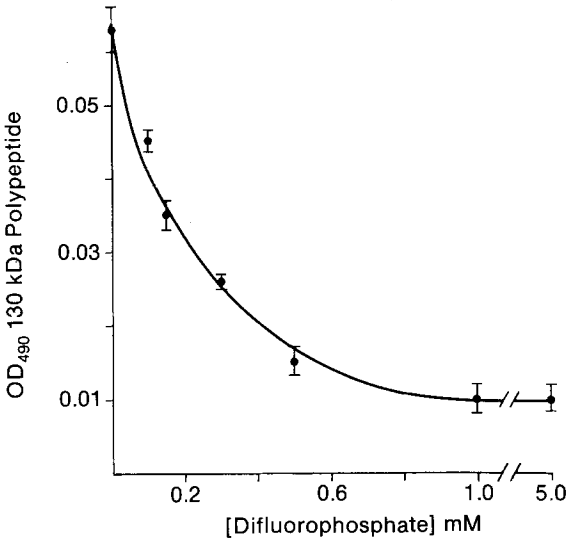

Fig. 7. Effect of [difluorophosphate] on FITC-PG labeling of 130-kDa polypeptide. SDS-BBMV (250 μg) were pretreated with 0.2 mM phenylglyoxal in the presence of substrates, as described in Materials and Methods. The protein was then treated with 50 μM FITC-PG in the presence of 50 mM potassium borate, pH 7.4, 100 mM NaCl and difluorophosphate. Difluorophosphate concentration was varied from 0.1 to 5 mM. SDS-Page was performed using 200 μg of protein as described in Materials and Methods. Individual sample tracks were examined for FITC-PG absorbance at 490 nm. Results are representative of three separate experiments

sures of Na^+-dependent phosphate uptake. Labeled phosphate uptake into SDS-BBM vesicles was determined using established methods. Phosphate uptake as measured by [^{32}P] phosphate does not distinguish between electrogenic or electroneutral uptake. The second method of uptake used was the generation of a membrane potential in Na^+ upon addition of phosphate. This measure of Na^+-dependent phosphate uptake only responds to electrogenic phosphate uptake.

The activation of phosphate uptake by Na^+ at pH 7.4 has similar $K_{0.5}$'s and Hill coefficients regardless of the technique employed to monitor phosphate uptake. [^{32}P] phosphate uptake displays a $K_{0.5}$ for Na^+ of 28 mM and a Hill coefficient of 1.9 in agreement with previous values [1, 4, 5]. When phosphate uptake is monitored using fluorescence enhancement of the carbocyanine dye, $diSC_3(5)$, the $K_{0.5}$ for Na^+ is 25 mM and the Hill coefficient is 1.6 (Table 1).

A Na^+-induced quenching of FITC-PG fluorescence was also observed in SDS-BBMV. The binding of FITC-PG at or near the cotransporter phosphate site has been described [14]. The $K_{0.5}$ for Na^+-induced conformational change was 25 mM consistent with the $K_{0.5}$ for Na^+ activation of phosphate uptake. A Hill plot of the FITC-PG fluorescence quenching as a function of Na^+ concentration indicated a Hill coefficient of 1.6. These results confirm and extend previous reports [1–5] that two Na^+'s are transported per phosphate.

The nature of phosphate that is transported was examined using [^{32}P] phosphate uptake and phosphate-induced membrane depolarization. The apparent K_m for phosphate was 100 μM using Na^+-dependent [^{32}P] phosphate uptake, and 400 μM using phosphate-induced membrane depolarization. The good agreement between these two techniques is consistent with a single Na^+-dependent cotransporter contributing the bulk of the Na^+-dependent phosphate transport. These results also suggest that at pH 7.4 the cotransporter is electrogenic since a phosphate-induced membrane depolarization was observed.

A second series of experiments using the difluoro- and monofluoro-derivatives of phosphate as inhibitors of Na^+-dependent phosphate uptake is described in Table 2 and Figs. 5–7. Only difluorophosphate inhibited Na^+-dependent phosphate uptake at pH 7.4. Inhibition was similar whether [^{32}P] phosphate uptake or membrane depo-

larization was used as the measure of phosphate uptake. The inhibition by difluorophosphate appears to be competitive with respect to phosphate. In contrast, at pH 7.4, monofluorophosphate was not an inhibitor at concentrations below 1 mM, and inhibited approximately 30% at 5 mM. At pH 7.4, monofluorophosphate exists as both the monovalent and divalent forms, in approximately a 1 : 396 ratio [7]. The poor inhibition seen with monofluorophosphate indicates that divalent phosphate is a poor substrate for the cotransporter at pH 7.4.

FITC-PG has been used to label a 130-kDa polypeptide in a Na^+ plus phosphate-sensitive manner [13, 14]. On the basis of inhibition of Na^+-dependent phosphate uptake, substrate protection requirements, and reconstitution of partially purified cotransporter [13] this 130-kDa polypeptide has been suggested to be the intestinal Na^+/phosphate cotransporter. The effect of phenylglyoxal on phosphate-induced membrane depolarization in Na^+ is consistent with the tentative assignment of the 130-kDa polypeptide as the electrongenic Na^+/phosphate cotransporter (Fig. 6).

The ability of difluorophosphate to substitute for phosphate in protection of the 130-kDa polypeptide against labeling by FITC-PG is shown in Fig. 7. Difluorophosphate protected against FITC-PG labeling like phosphate with a $K_{0.5}$ of 234 ± 20 μM (n = 3). Protection by difluorophosphate was 97% of that seen with 1 mM phosphate. Monofluorophosphate did not protect the 130-kDa polypeptide at concentrations up to 5 mM. These results suggest that the 130-kDa polypeptide labeled by FITC-PG is an electrogenic Na^+/phosphate cotransporter.

The substrate stoichiometry of the intestinal Na^+/phosphate cotransporter is apparently 2, with $H_2PO_4^-$ the predominant substrate. This is suggested by the apparent K_m's for phosphate using isotope uptake and membrane depolarization, and inhibition of Na^+-dependent phosphate uptake by difluorophosphate but not monofluorophosphate at pH 7.4. There is some Na^+-dependent uptake, which is insensitive to 5 mM difluorophosphate at pH 7.4. This 5 to 20% of the Na^+-dependent uptake is sensitive if membrane depolarization is used as the measure of phosphate uptake. Membrane depolarization by phosphate was completely inhibited by 1 mM difluorophosphate at pH 7.4, while isotopic phosphate uptake was 85% inhibited. On this basis it appears that Na^+/phosphate cotransporter at pH 7.4 is approximately 90% electrogenic with $H_2PO_4^-$ as the transported phosphate species.

At this time, there is no way to distinguish between incomplete inhibition by difluorophosphate and two cotransporters. The similar inhibition patterns, transporter kinetics and labeling by FITC-PG, and substrate protection against labeling is consistent with a single electrogenic Na^+/phosphate cotransporter carrying two Na^+'s and $H_2PO_4^-$. However, a second electroneutral cotransporter or cotransport mode responsible for approximately 10% of the observed Na^+-dependent phosphate uptake at pH 7.4 cannot be completely ruled out.

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