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Intestinal Maturation: Characterization of Mitochondrial Phosphate Transport in the Rat and its Regulation by 1,25-$(OH)_2$ Vitamin D_3

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ABSTRACT. The mitochondria play a major role in the regulation of oxidative phosphorylation within the cell. Despite the fact that the enterocytes receive the majority of absorbed phosphate and their high metabolic turnover rate, the role of the intestinal mitochondria in phosphate transport system during maturation is not known. Therefore, the current studies were designed to characterize phosphate transport by jejunal mitochondria of rats during maturation (suckling, weanling, and adolescent rats). The functional integrity of the intestinal mitochondria of suckling and adolescent rats was determined by oxygen consumption studies demonstrating respiratory control ratios of more than 3 when succinate was used as a test substrate. Phosphate uptake was significantly stimulated by the presence of 3 mM ATP at all age groups studied. Maximal phosphate uptake in the presence of 3 mM ATP and 2 mM succinate was 16.5 ± 1.0, 20.5 ± 1 and 28.7 ± 0.4 nmol/mg protein (mean ± SE) in suckling, weanling, and adolescent rats respectively. ATP-dependent phosphate uptake was inhibited by 80% with 100 μM p-MB. Kinetic parameters for ATP stimulated phosphate uptake at 10 s revealed a Km of 4 ± 0.9, 2.8 ± 0.4, and 0.9 ± 0.1 mM and V_{max} of 5 ± 0.7, 9.5 ± 1, and 11 ± 0.7 nmol/mg protein per 10 s in suckling, weanling, and adolescent rats, respectively. Phosphate uptake was also stimulated by an inwardly directed pH gradient (pH out < pH inside) compared to no pH gradient condition suggesting the presence of PO_4^-/OH^- exchange. The kinetic of the PO_4^-/OH^- exchange showed a Km of 1.9 ± 0.4 and 2.2 ± 1 mM and a V_{max} of 1.3 ± 0.4 and 4.4 ± 1.5 nmol/mg protein/10 s for suckling and adolescent rats, respectively. Vitamin D deficiency decreased markedly the V_{max} and Km of ATP-dependent phosphate uptake in adolescent rats to 1.6 ± 0.2 nmol/mg protein/10 s and 0.2 ± 0.1 mM, respectively. 1,25-$(OH)_2$ vitamin D_3 administration, 8 h before death increased the V_{max} to 3.06 ± 0.8 ($p < 0.01$) without a significant change in the Km. These results indicate that phosphate uptake into the mitochondria occurs by a ATP-dependent and PO_4^-/OH^- exchange mechanisms. The ATP-dependent mechanism is regulated by 1,25-$(OH)_2$ vitamin D_3. (*Pediatr Res* 25:605–611, 1989)

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Abbreviations

DEAE-cellulose, anion exchange cellulose, diethyl aminoethyl cellulose
HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
PMSF, Phenylmethylsulfonyl fluoride
p-MB, *p*-chloromercuribenzoic acid

The mitochondria occupy a substantial fraction of the cytoplasm of virtually all eucaryotic cells and their role in oxidative metabolism is well established (1–3). The net transport of inorganic phosphate into the mitochondria matrix is essential for maintenance of steady state oxidative phosphorylation. A specific transporter has been purified and reconstituted from beef heart (3). Similarly, a highly specialized transport system from liver mitochondria has been well characterized (4). Despite the fact that the intestinal epithelial cell receives the majority of transported phosphate, the role of the intestinal mitochondrial phosphate transport system has not been examined. Furthermore, to our knowledge, the developmental characteristics of intestinal mitochondria phosphate transport system are entirely unknown.

Until recently, the preparation of mitochondria from enterocytes was difficult because the small intestine contains a large number of goblet cells (5), which secret mucus, thus complicating the isolation of intestinal mitochondria. In the present study, rat jejunal mitochondria were obtained using DEAE-cellulose which completely binds and removes the intestinal mucus. This method was adapted from that of Lawrence and Davies (6). Our results characterize for the first time, intestinal mitochondrial phosphate transport system and examine the maturational aspects of this process. A millipore filtration technique was used to study mitochondrial phosphate transport (7, 8).

MATERIALS AND METHODS

Two male adolescent rats (42 d old), 8–10 weanling rats (21 d old), and 8–10 suckling rats (15 d old) of the Sprague-Dawley strain were used for each experiment. Rats were obtained from Harlan Laboratories (Indianapolis, IN). Adolescent and weanling rats were fed a standard diet (Ca 1.2%, P 0.8%, vitamin D 1700 IU/g) (Teklad Diets, Teklad Co., Madison, WI). Suckling rats were allowed to suckle mother's milk freely. Vitamin D deficiency in adolescent rats was induced by feeding a vitamin D-deficient diet (Ca 0.4%, P 0.3%, no vitamin D) to weanling rats for 4 wk while being kept in the dark. 1,25-$(OH)_2$ vitamin D_3 (Calcijex, Abbott Laboratories, N. Chicago, IL) 100 pg/100 g body weight was given intraperitoneally to vitamin D-deficient

rats whereas controls received a buffered normal saline 8 h before death.

DEAE cellulose and other reagents and biochemicals were purchased from either Sigma Chemical Co. (St. Louis, MO), Fisher Scientific (Norcross, CA), or Aldrich Chemical Company (Milwaukee, WI). Nylon cloths for the filtration of mucosal samples were obtained from general nylon goods. ^{32}P was obtained from New England Nuclear (Boston, MA). Cellulose nitrate filters, 0.45 μm pore size, were obtained from Sartorius Filters, Inc. (Hayward, CA).

Rat jejunal mitochondria were prepared by slight modification of the method of Lawrence and Davies (6), in which an anion-exchange cellulose (DEAE-cellulose) was used to remove adherent mucus. After adequate anesthesia with intraperitoneal injection of pentobarbital (200 mg/kg), the abdominal cavity was opened. The jejunal segment was isolated, cannulated, and then flushed out with ice cold "gut wash" containing: 160 mM sucrose, 110 mM mannitol, 2 mM HEPES, 3.3 mM Tris, 0.25 mM EGTA, 0.1 mM PMSF, pH 7.4, which was oxygenated with 100% O_2 for at least 30 min before use *in situ*. The jejunum was then removed and placed in the same medium "gut wash." The jejunum was then everted and dried by filter paper. Mucosal scrapings were obtained by glass slides on a cooled glass plate. Amounts of mucosal scrapings obtained were 2.5–3 g for two adolescent rats, 2–2.5 g for weanling rats, and 1.5–2 g for suckling rats.

Different amounts of the anion exchange cellulose solution was prepared for each age group (3 g DEAE-cellulose for adolescent, 2 g for weanling, and 1.5 g for suckling rats). This solution consisted of 3 g DEAE-cellulose, 7500 U/100 ml heparin, 1 mM dithiothreitol as final concentrations, 30 ml isolation medium A (70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.5 mM EGTA, 0.1 mM PMSF, 0.37 g/100 ml BSA fatty acid free fraction V, pH 7.4). The mucosal scrapings were stirred to an almost homogenate state with each amount of DEAE-cellulose solution. After standing 2 min, 40 ml (30 ml for weanling, suckling rats) isolation medium A was added to the suspension, stirred, and then homogenized by six strokes using glass-Teflon homogenizer. The homogenate was then diluted with 50 ml (40 and 30 ml for weanling and suckling rats, respectively) of isolation medium A and re-homogenized by six strokes using glass-Teflon homogenizer. The homogenate was then centrifuged at 750 × g for 10 min at 4° C in a refrigerated centrifuge (Beckman Instruments, Inc., Palo Alto, CA, model J2-21). Supernatant was then decanted and filtered through nylon-cloth and re-centrifuged at 10 000 × g for 7 min. Crude mitochondria pellet was obtained, which had a mucus layer above it. The layer of mucus was scraped off with a pipette.

The mitochondrial rich fraction was resuspended with 35 ml of isolation medium A and re-centrifuged at 14 000 × g for 7 min. The resultant pellet was resuspended with 35 ml of isolation medium B (70 mM sucrose, 200 mM mannitol, 2 mM HEPES, 1.2 g/100 ml BSA, pH 7.2) and then centrifuged at 14 000 × g for 10 min. The resultant pellet was washed twice to remove bovine albumin by 35 ml of washing solution (290 mM mannitol, 2 mM HEPES, pH 7.2) using centrifugation at 14 000 × g for 7 min. The mucus layer above the mitochondria-rich fraction was removed at each step. The final pellet was resuspended with 1.0–1.3 ml washing solution for phosphate studies.

PO_4^- transport was measured by a rapid filtration technique (7, 8). All experiments were performed at 25° C. The amount and composition of the incubation media for each experiment is described in the figure legends in the "Results." PO_4^- influx reaction was stopped by a 1 ml ice cold stop solution consisting of 100 mM NaCl, 100 mM mannitol, 20 mM HEPES, 5 mM KH_2PO_4, pH 7.2, and 0.1 mM p-MB. Separation of mitochondrial vesicles from the incubation solution were achieved by millipore filtration (0.45 μm pore size, Sartorius Filters, Inc., Hayward, CA). The filters were washed by a 5-ml ice cold stop solution and placed in a scintillation vial. Radioactivity was determined in a liquid scintillation counter (Beckman Instruments) using Scint Verse II (Fisher Scientific, Norcross, CA). Protein determinations were performed by the method of Lowry *et al.* (9).

To characterize the purity of our preparation, transmission electronmicroscopic and enzyme marker studies were done. Enzyme markers were determined in mucosal crude homogenate and mitochondrial pellets. Cytochrome c oxidase, a marker for mitochondria, was measured by the method of Beaufy *et al.* (10). Leucine aminopeptidase, a marker for brush border membranes, was measured by a Sigma kit; Na^+-K^+-ATPase, a marker for basolateral membranes, by the method of Scharschmidt *et al.* (11); NADPH cytochrome c reductase, a marker for endoplasmic reticulum, by the method of Beaufy *et al.* (10); galactosyl transferase, a marker for Golgi apparatus, by the method of Moore *et al.* (12). The functional integrity of the mitochondrial preparation was determined by oxygen consumption studies using an oxygen monitor (YSI biological oxygen monitor, Yellow Instrument Co., Yellow Springs, OH), fitted with a clark type oxygen electrode. Rates of oxygen consumption were calculated according to Chance and Williams (13). Succinate was used as a respiratory substrate. Rates of oxygen consumption were also monitored with the addition of ADP (state 3) and after depletion of exogenous ADP (state 4). The respiratory control ratios state 3/state 4 respiration, an indicator of mitochondrial functional integrity was calculated as well as the ADP/O ratios.

All values were expressed graphically as the mean ± 1 SE. Student's *t* test was used to evaluate the statistical significance of differences between the groups. A probability value ($p < 0.05$) was considered statistically significant.

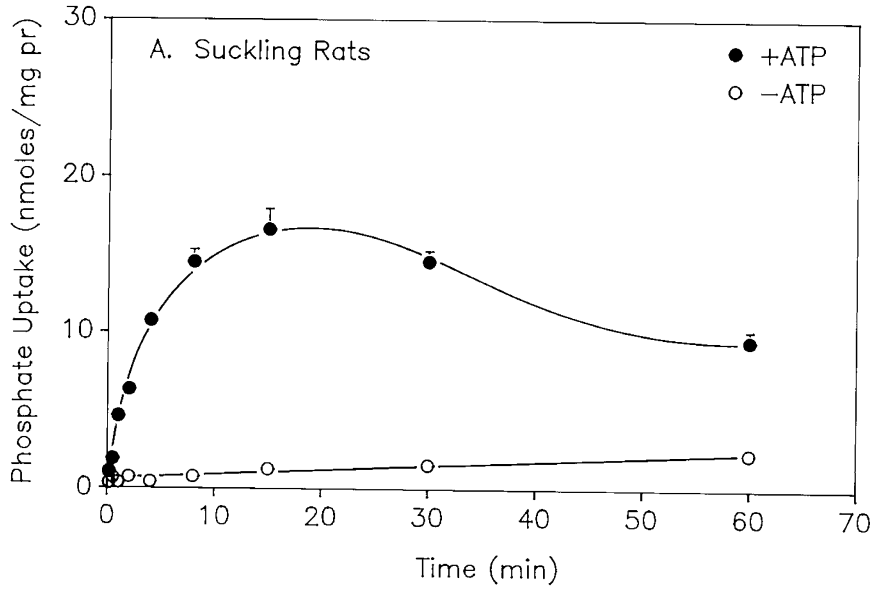
RESULTS

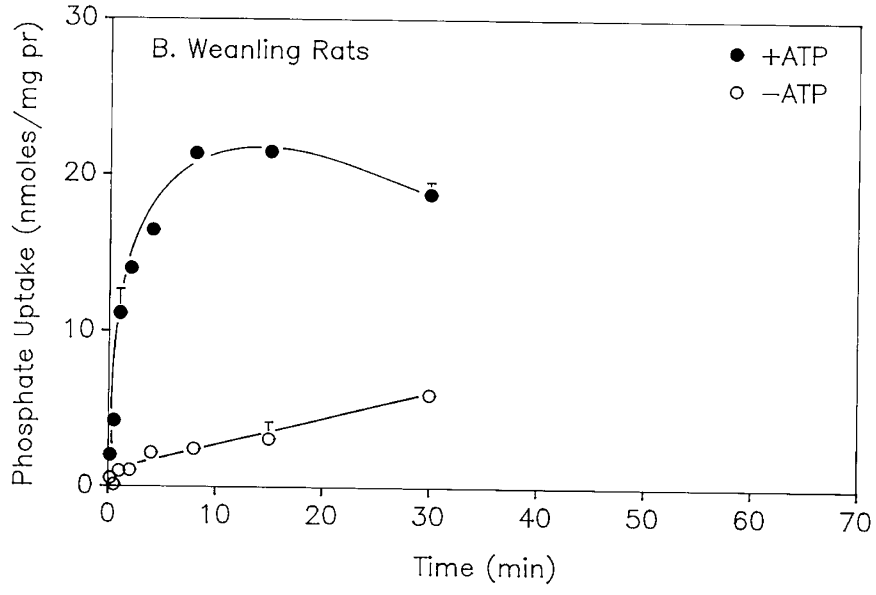
Purity of mitochondrial preparation. Highly enriched energized mitochondria were obtained from all age groups as determined by transmission electronmicroscopy. An enriched mitochondrial preparation was evident by the 12-fold enrichment in cytochrome c oxidase. There was minimal contamination with endoplasmic reticulum, whereas marker enzymes for brush borders, basolateral membranes, and Golgi apparatus were impoverished. Respiratory control ratios were 5.1 ± 0.4 and 3.9 ± 0.4 in both adolescent and suckling rat mitochondria. ADP/O ratio was 1.80 ± 0.1 and 1.9 ± 0.2 for adolescent and suckling rats respectively suggesting functional mitochondria (14).

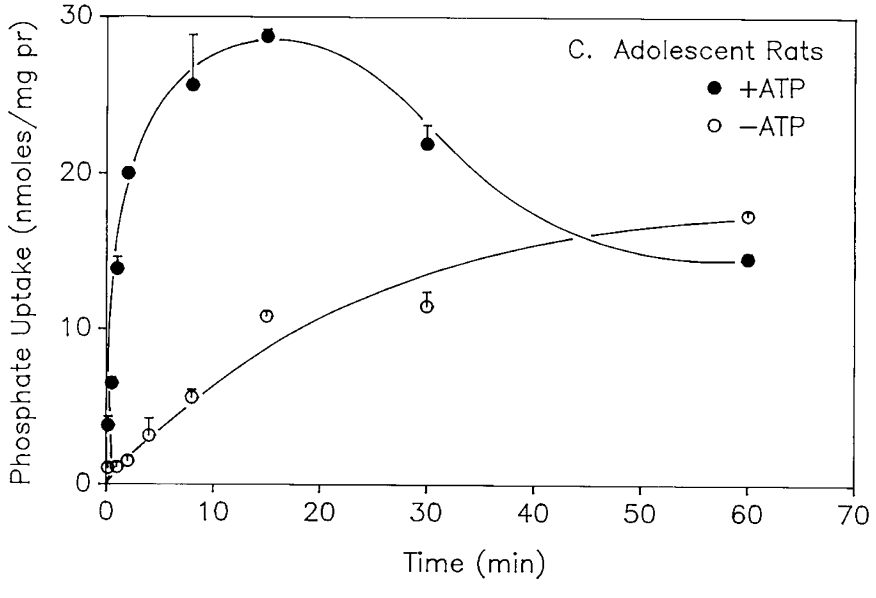
Vitamin D status of animals. Serum concentration of 1,25-$(OH)_2$ vitamin D_3 in control rats was 157 ± 2 pg/ml, whereas in vitamin D-deficient rats,the value was 41 ± 10 pg/ml. Administration of 1,25-$(OH)_2$ vitamin D_3 increased serum levels to 192 ± 12 pg/ml.

PO_4 uptake with time. A 20 μl of mitochondrial suspension was diluted in a 60-μl incubation media containing in final concentration, 120 mM KCl, 110 mM mannitol, 2 mM succinate (K-salt), 0.6 mM sucrose, 3 mM ATP-Mg^{++}, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.2, 1 mM phosphate and ^{32}P (0.25 μCi/tube) which had already been dried. Figure 1 shows PO_4^- uptake in the presence and absence of 3 mM ATP. Phosphate uptake in

Fig. 1. Time course of phosphate uptake in the presence and absence of ATP in suckling, weanling, and adolescent rats. Intestinal mitochondria were preloaded with 290 mM mannitol, 2 mM HEPES, p 7.2. Incubation was started by the addition of mitochondria to a media containing in final concentration either 120 mM KCl, 100 mM mannitol, 10 mM HEPES/Tris, 5 mM $MgCl_2$, 3 mM ATP-Mg^{++}, 2 mM succinate (K-salt), 1 mM phosphate, and tracer ^{32}P or 120 mM KCl, 110 mM mannitol, 10 mM HEPES/Tris, 5 mM $MgCl_2$, 2 mM succinate (K-salt), 0.6 mM sucrose buffer, pH 7.2, 1 mM phosphate and tracer ^{32}P. Reaction was stopped at 10, 30 s, 1, 2, 4, 8, 15, 30, and 60 min. The composition of the stop solution was 100 mM mannitol, 100 mM NaCl, 20 mM HEPES/Tris, 5 mM KH_2PO_4, 0.1 mM p-MB.


A. Suckling Rats
+ATP
−ATP
Phosphate Uptake (nmoles/mg pr)
Time (min)
0
10
20
30
40
50
60
70


B. Weanling Rats
+ATP
−ATP
Phosphate Uptake (nmoles/mg pr)
Time (min)
0
10
20
30
40
50
60
70


C. Adolescent Rats
+ATP
−ATP
Phosphate Uptake (nmoles/mg pr)
Time (min)
0
10
20
30
40
50
60
70

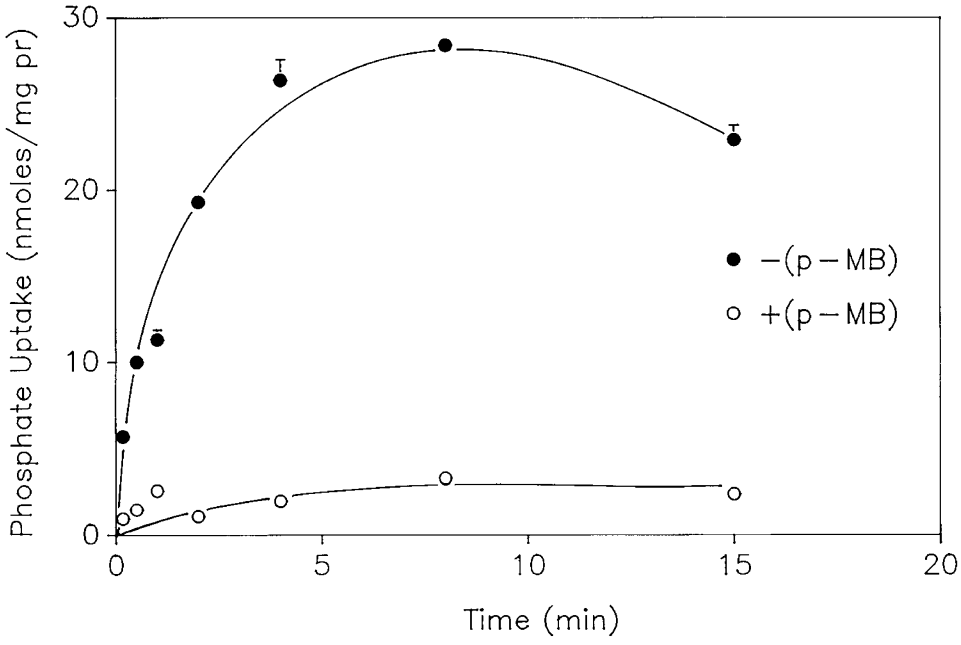


Fig. 2. Effect of p-MB on phosphate uptake. Mitochondria were preloaded with 290 mM mannitol, 2 mM HEPES, pH 7.2. Incubation was started by the addition of 20 μl of mitochondria to an incubation media containing in final concentration 120 mM KCl, 110 mM mannitol, 10 mM HEPES/Tris, 5 mM $MgCl_2$, 3 mM ATP-Mg^{++}, 2 mM succinate (K-salt), 0.6 mM sucrose buffer pH 7.2, 1 mM phosphate, and tracer ^{32}P. *Open circles* indicate phosphate uptake in the presence of p-MB (100 μM).

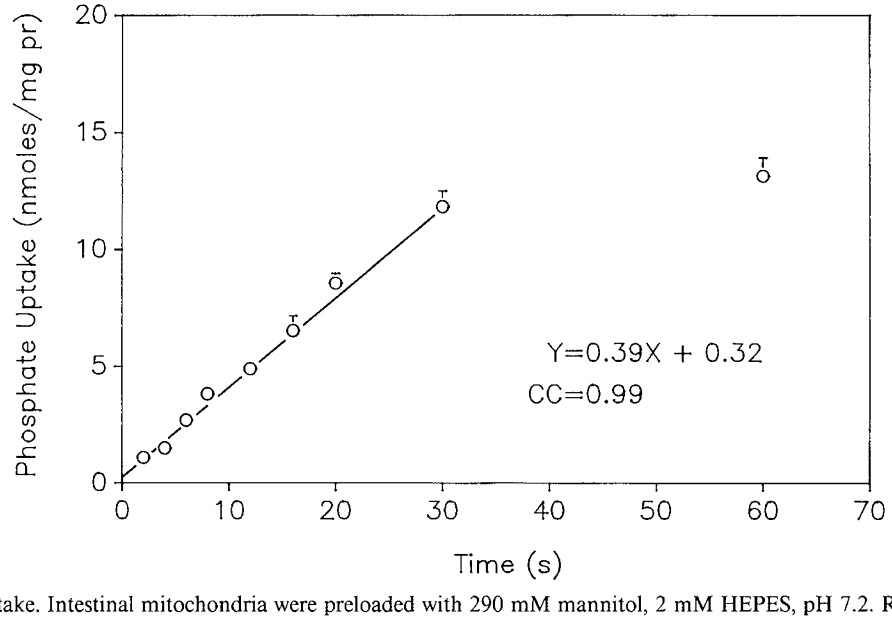


Fig. 3. Initial rate uptake. Intestinal mitochondria were preloaded with 290 mM mannitol, 2 mM HEPES, pH 7.2. Reaction was started by the addition of 20 μl of mitochondria to an incubation media containing in final concentration, 120 mM KCl, 110 mM mannitol, 10 mM HEPES/Tris, 5 mM $MgCl_2$, 3 mM ATP-Mg^{++}, 2 mM succinate (K-salt), 0.6 mM sucrose, buffer pH 7.2, 1 mM phosphate, and tracer ^{32}P. Phosphate uptake was determined with time up to 60 s. The uptake appears linear up to 30 s.

the presence of ATP was significantly greater than in the absence of ATP (p, < 0.05–0.001). PO_4^- uptake was inhibited by more than 80% by the addition of 100 μM p-MB (Fig. 2).

Kinetics of PO_4^- uptake with age. Initial uptake rate was determined at 2, 4, 6, 8, 12, 16, 20, 30, and 60 s. Figure 3 shows PO_4^- uptake in the presence of 3 mM ATP up to 60 s in adolescent rats. Initial rate uptake was linear up to 30 s. Similar findings were seen in suckling and weanling rats. Therefore, kinetic studies were done at 10 s, well within the linear range of uptake. PO_4^- uptake was determined using different PO_4^- concentrations (0.05 to 5 mM). Km and V_{max} were calculated using a computerized model of the Michaelis-Menten kinetics (15).

Figure 4 depicts the kinetic parameters for PO_4^- uptake in mitochondria of jejunum at all age groups. Km values were 4.03 ± 0.95, 2.79 ± 0.41, and 0.94 ± 0.19 mM and V_{max} values were 5.03 ± 0.70, 9.44 ± 1.14, and 10.71 ± 0.77 nmol/mg protein/10 s for suckling, weanling, and adolescent rats, respectively. Km and V_{max} values of suckling and adolescent rats were significantly different (p < 0.05–0.01). Therefore, the affinity and capacity of the mitochondrial phosphate transport system increases with advancing age.

Mitochondrial phosphate/hydroxyl exchange. Figure 5 shows the effect of inwardly directed pH gradient on phosphate uptake. Phosphate uptake was stimulated with inwardly directed pH gradient (pH inside 7.5, pH outside 6.0) compared to no pH gradient conditions of either (pH inside 7.5 = pH outside 7.5 or pH inside 6.0 = pH outside 6.0). The kinetics of this exchanger in adolescent rats showed a V_{max} of 8.9 ± 0.8 nmol/mg protein/

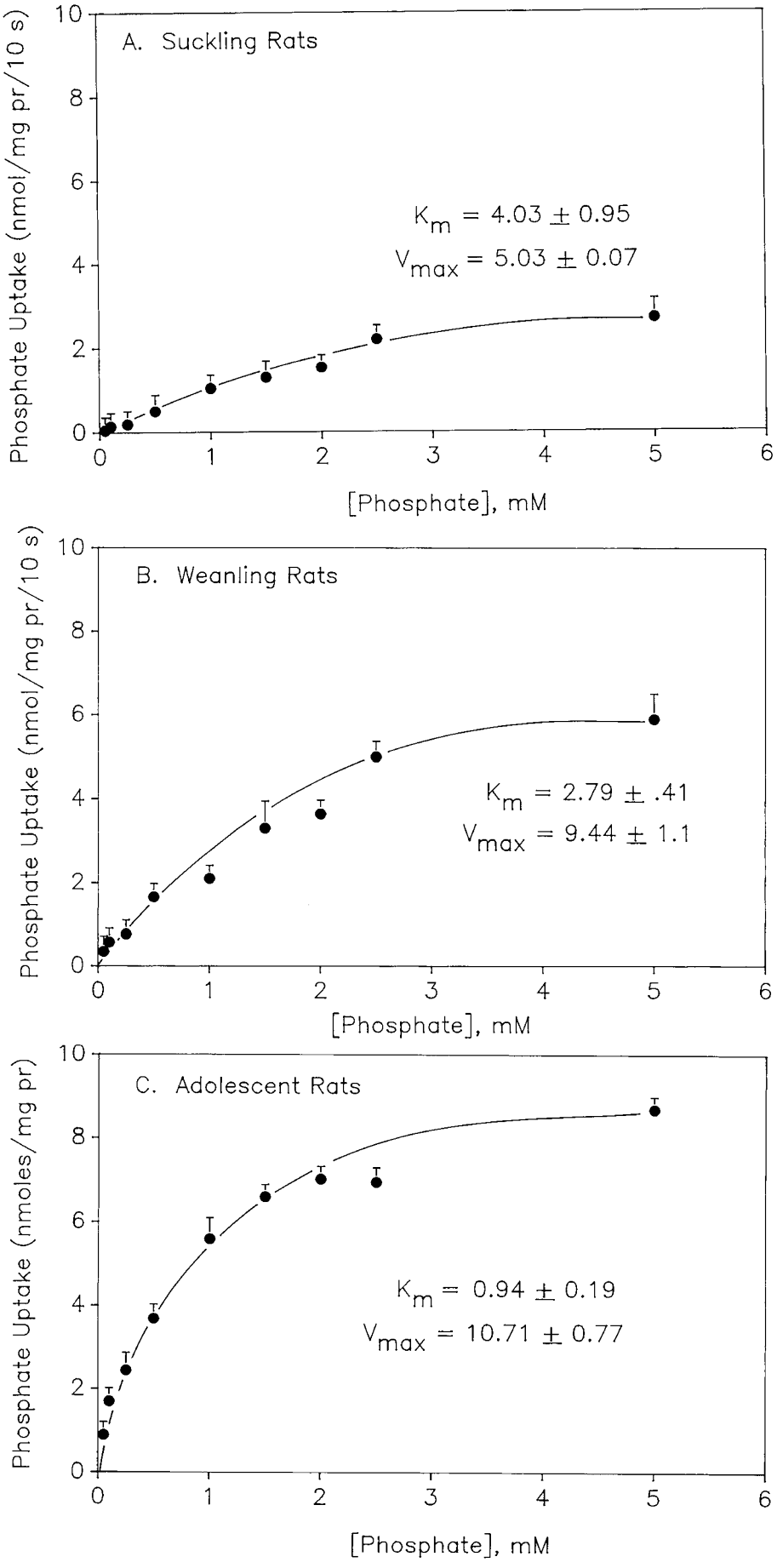


Fig. 4. Kinetics of phosphate uptake in suckling, weanling, and adolescent rats. Intestinal mitochondria were preloaded with 290 mM mannitol, 2 mM HEPES, pH 7.2, incubated in a media containing in final concentration 120 mM KCl, 110 mM mannitol, 10 mM HEPES/Tris, 5 mM $MgCl_2$, 3 mM ATP-Mg^{++}, 2 mM succinate (K-salt), 0.6 mM sucrose, buffer pH 7.2, different phosphate concentrations 0.5–5 mM, and tracer ^{32}P. Uptake was determined at 10 s. Kinetic parameters were obtained using a computerized model of the Michaelis-Menten kinetics.

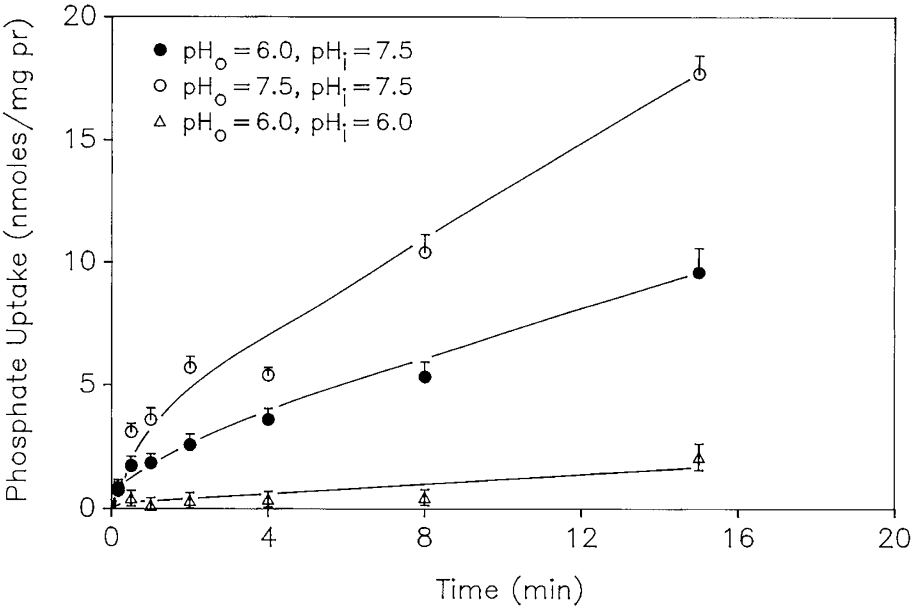


Fig. 5. Phosphate/OH^- exchange. Intestinal mitochondria were preloaded with 270 mM mannitol, 20 mM HEPES/Tris, pH 7.5, or 270 mM mannitol, 20 mM 2-(N-morpholino)ethanesulfonic acid buffer pH 6.0. Incubation was started by the addition of mitochondria to a media containing in final concentration either 120 mM KCl, 100 mM mannitol, 20 mM HEPES, 5 mM $MgCl_2$, 5 mM succinate (K-salt), 0.6 mM sucrose buffer pH 7.5, 1 mM phosphate concentration, and tracer ^{32}P, or 120 mM KCl, 100 mM mannitol, 20 mM HEPES, 5 mM $MgCl_2$, 5 mM succinate (K-salt), 0.6 mM sucrose buffer pH of 7.5 or 6.0, 1 mM phosphate, and tracer ^{32}P. Reaction was stopped at 10 s, 30 s, 1, 2, 4, 8, 15, 30, and 60 min. The *open circles* indicate phosphate uptake under outwardly directed (^-OH) gradient ($pH_0 = 6$, $pH_i = 7.5$). The *closed circles* indicate phosphate uptake under no pH gradient conditions ($pH_0 = 7.5$, $pH_i = 7.5$). The *open triangles* indicate phosphate uptake under no pH gradient condition of ($pH_0 = 6$, $pH_i = 6$).

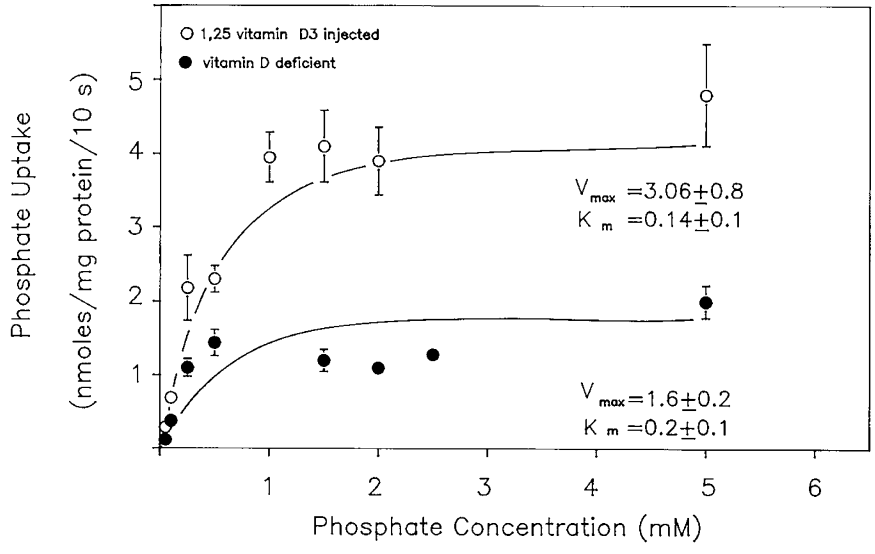


Fig. 6. Kinetics of phosphate uptake in adolescent vitamin D-deficient and 1,25-$(OH)_2$ D_3-injected rats. The experimental procedure is similar to that of Figure 4. Kinetic parameters were obtained using a computerized model of the Michaelis-Menten kinetics. V_{max} is significantly greater in the injected group compared to deficient group ($p < 0.01$).

10 s and a Km of 0.41 ± 0.06 mM. The Km is significantly different from the Km with (pH inside 7.5, pH outside 7.5) $p < 0.01$.

Role of 1.25-$(OH)_2$ vitamin D_3 in PO_4 uptake. To determine whether vitamin D_3 has a role in regulating phosphate uptake by the mitochondria, we administered 1,25-$(OH)_2$ vitamin D_3 to vitamin D-deficient adolescent rats. Another group of vitamin D-deficient rats received the vehicle of 1,25-$(OH)_2$ vitamin D_3. Kinetic analysis of phosphate uptake in the presence of 3 mM ATP showed a significant decrease in V_{max} and Km 1.6 ± 0.2 nmol/mg protein/10 s and 0.2 ± 0.1, respectively, in vitamin D-deficient rats. 1,25-$(OH)_2$ vitamin D_3 administration increased the V_{max} to 3.06 ± 0.8 nmol/mg protein/10 s ($p < 0.01$ compared to V_{max} of vitamin D-deficient rats), without a significant change in the Km (Fig. 6).

DISCUSSION

The mitochondria play a major role in the oxidative phosphorylation process within the cell. A well-described phosphate transport system has been characterized in rat liver mitochondria (4). The protein responsible for the transport of phosphate has

been purified from liver, heart, and muscle (2, 4). To our knowledge, the transport of phosphate across the mitochondria of the enterocyte has not been described. This is surprising in view of the rapid rate of proliferation of the intestinal enterocyte and the fact that the enterocyte receives the body's requirement for phosphate from the intestinal lumen. Until recently, it has been difficult to isolate intestinal mitochondria because of the difficulty of removing the adherent mucus. We used an anion exchanger (cellulose) to remove the adherent mucus according to the method of Lawrence and Davies (6). Using this technique, we obtained a highly enriched energized intestinal mitochondria of rats of various ages during development. Phosphate uptake was ATP-dependent at all age groups. Maximal phosphate uptake in the presence of 3 mM ATP and 2 mM succinate at 1 mM phosphate was obtained at 10–15 min. Maximal phosphate uptake increased with advancing age. Phosphate uptake in the absence of ATP was 10 to 30% of that in the presence of ATP at all age groups studied. p-MB, a mercurial agent and a known inhibitor of phosphate uptake by liver mitochondria, also inhibited phosphate uptake by intestinal mitochondria. The inhibition by p-MB suggests that the transport component has an essential sulfahydryl group. The kinetic parameters reveal a higher capacity, high affinity transport system in adolescent rats while in suckling rats, a low capacity low affinity system is present. Weanling rats' values were intermediate between suckling and adolescent rats. The reasons for these changes with age may be related to greater ATPase activity in the adolescent rats mitochondria compared to corresponding mean values in suckling rats mitochondria (14). The second mechanism for phosphate transport is supported by PO_4^-/OH^- exchange. This is evident by marked stimulation of phosphate uptake by the inwardly directed pH gradient compared to no pH gradient condition. The observation that PO_4 uptake was lower at $pH_i = 6$, $pH_0 = 6$ compared to $pH_0 = 6.0$ pH 7.5, suggests that the media pH per se is not the stimulator for the uptake rather the $-OH$ gradient provides the driving force for PO_4 uptake. The possibility of PO_4^-/H^+ cotransport cannot be ruled out by the present studies. The kinetics of this hydroxyl exchange system shows a Km significantly lower than corresponding values obtained in the presence of ATP; suggesting that the pO_4^-/OH^- exchange system has a higher affinity to transport PO_4^- compared with that supported by the hydrolysis of ATP.

To our knowledge, the role of vitamin D in phosphate uptake by subcellular organelles has never been investigated. Therefore, it was of interest to determine whether 1,25-$(OH)_2$ vitamin D_3, the most active polar metabolite of vitamin D, plays a role in regulating PO_4 uptake by the intestinal mitochondria. Our data show clearly that vitamin D deficiency results in 4-fold decrease in V_{max} of PO_4 uptake, whereas 1,25-$(OH)_2$ D_3 administration restored partially V_{max} of PO_4 uptake.

Therefore, the current studies provide evidence for vitamin D regulated specialized mechanisms for phosphate uptake by the intestinal mitochondria. These processes show developmental patterns in regard to their kinetic parameters.

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