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Phosphate Uptake by Syncytial Brush Border Membranes of Human Placenta

MICHÈLE G. BRUNETTE AND SERGE ALLARD

Department of Pediatrics and Research Center, Maisonneuve Hospital and University of Montreal, Quebec, Canada

ABSTRACT. Human placental brush border membranes were obtained from full-term normal vaginal deliveries. A sodium dependent transport system for phosphate was identified in these membranes. In the presence of a sodium gradient, at pH 7, T° 35° C, the apparent Km and V_{max} were 86.5 ± 24 μM and 1.9 ± 0.18 nmol mg^{-1} 30 s^{-1}. The apparent Km was pH dependent, increasing to 500 ± 55 μM when the pH was elevated to 8.5. At low substrate concentration, the optimal uptake was obtained at pH 7.0. Both monovalent and divalent forms of phosphate were transported so that alteration in the transport by pH is believed to be the result of a direct action on the transport system. Finally, sodium levels in the incubation medium dramatically influenced phosphate uptake by increasing the maximal velocity without modifying the affinity for the substrate. This active transport through the placental brush border membranes is believed to be at the origin of the maternal-fetal gradient of phosphate observed during pregnancy (*Pediatr Res* 19: 1179–1182, 1985)

Abbreviations

BBM, brush border membranes
PO_4, serum phosphate

Under normal circumstances, bone mineral content in the fetus increases exponentially throughout gestation and is in linear correlation with body weight (1–3). Two-thirds of the total body phosphorus in term neonates is deposited during the 3rd trimester (4). Since fetal PO_4 levels are significantly higher than maternal levels (2, 5, 6), phosphate ions are thought to be transported actively across the placenta and, specifically, through the maternal membrane of the trophoblast.

Whole placenta perfusion experiments revealed that PO_4 transport from maternal to fetal bed is dependent upon the concentration of Na in the perfusate (7, 8). It is therefore probable that in the placenta as in the intestine and the kidney, PO_4 is cotransported with Na through the syncytial plasma BBM.

To further our knowledge of the process involved in this Na-dependent transport of PO_4 through the placenta BBM, we studied the kinetics of PO_4 uptake by BBM vesicles prepared from human placentas, as well as the influence of pH and Na concentration upon this uptake.

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Reprint address Michèle Gagnan-Brunette, M.D., Maisonneuve-Rosemont Hospital, 5415 l'Assomption Boulevard, Montreal, P.Q., H1T 2M4, Canada.
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MATERIALS AND METHODS

Membrane preparation. BBM were prepared using a modified version of the method described by Smith *et al.* (9). The placentas obtained from full term normal vaginal deliveries were emptied of blood by perfusion of 1 liter of 10 mM Tris-270 mM mannitol pH 7.5 through the umbilical artery ramifications. Approximately 150 to 200 g of placental tissue obtained from the central part of the placenta was cut into 4–6 mm slices, placed in ice-cold Tris-mannitol buffer and gently stirred with a magnetic bar at 4° C for 30 min.

The tissue and medium were then passed through a coarse cotton gauze. The filtrate was centrifuged at 100 000 × *g* for 60 min. The pellet was separated from red blood cells, homogenized with a Potter homogenizer at 2000 rpm, resuspended in 15 ml Tris-mannitol buffer with 10 mM $MgCl_2$ and again stirred with a magnetic bar (4° C for 20 min). The suspension was then centrifuged at 3000 × *g* for 10 min. The supernatant was decanted and centrifuged at 27 000 × *g* at 4° C for 10 min. The loosely packed white layer was gently washed at this point and resuspended in 10 mM Tris-mannitol, giving a final protein concentration of approximately 8 mg/ml. Unless specified, the pH of the membrane suspension was 7.5. In pH experiments, the pH of this suspension varied from 6.5 to 8.5. Uptake experiments were started after a 1-h preincubation period in order to obtain equilibrium between the inside and the outside media of the vesicles. Whole placental homogenate was prepared from washed placental samples by simple homogenization.

Marker enzyme analysis. Alkaline phosphatase was determined by measuring *p*-nitrophenol after hydrolysis of *p*-nitrophenyl phosphate (10). Na-K-ATPase activity was measured by determining free phosphate following incubation with media containing 100 mM Tris-HCl pH 7.8, 6 mM ATP, 6 mM $MgCl_2$ and 50 mM NaCl, 5 ml KCl with or without 1 mM ouabain (11). Succinate dehydrogenase activity was determined using Pennington's method (12).

The mean enzyme activity for homogenates and membrane preparations was: Alkaline phosphatase: 7.1 ± 0.7 and 189.0 ± 10.1 μmol PO_4 mg^{-1}, 15 min^{-1}, (enrichment × 27), Na-K-ATPase 0.05 ± 0.01 and 0.33 ± 0.04 μmol, mg^{-1}, 15 min^{-1} (enrichment × 6) and succinate dehydrogenase 0.97 ± 0.15 and 0.29 ± 0.06 nmol, mg^{-1}, 15 min^{-1}, (enrichment × 0.3), respectively. The relatively high enrichment in Na-K ATPase is probably due to the fact that the placenta consists, for the most part, of vessels, blood, and conjunctive tissue, *i.e.* in structures devoid of Na-K ATPase. Then the homogenate prepared from the whole tissue is particularly poor in this enzyme and the slightest contamination of the BBM preparation with basal membrane results in an apparently high enrichment in ATPase. This is also the case but to lesser degree for alkaline phosphatase which is present in plasma and white blood cells. The relative purity of the BBM suspension was confirmed by electronic microscopy, which

showed vesicles with variable size but homogeneous structure of the membrane, identical to those reported by Smith *et al.* (9).

^{32}P phosphate uptake. Phosphate uptake was measured using the Millipore filtration technique initially described by Hoffman *et al.* (13) for renal BBM preparations. Aliquots of 5-μl membrane suspension, *i.e.* approximately 40 μg protein, were incubated in 25 μl of a medium containing (mM final concentration): 100 mannitol, 100 NaSCN (or KSCN) 10 Tris-HEPES at the specified pH, and from 0.04 to 3.0 ^{32}P Na_2HPO_4 (New England Nuclear, Boston, MA). Incubation was at 35° C, for 30 s, unless otherwise specified. It was terminated by the addition of 1 ml of cold "stop solution" containing 100 mM mannitol, 5 mM Tris-HEPES at the same pH as the incubation medium and 100 mM choline chloride. The suspension was filtered through a 0.45-μm Millipore filter and rinsed with an additional 5 ml of stop solution. The filters were dried and the radioactivity was assayed in 10 ml Insta-gel (Packard Instrument, Downers Grove, IL). In each experiment, the nonspecific absorption to the BBM and the filters (less than 0.15%) was estimated by measuring the PO_4 uptake at time zero in an incubation medium containing K instead of Na.

Chemical measurement. The protein content of the membrane suspensions or homogenates was determined by spectrophotometry at 280 nm in 1% sodium dodecyl sulfate, using a standard curve previously established by measuring chemically the protein concentration (14) concomitantly with the absorbance of the same membrane suspension in SDS at the above wavelength. All transport data are reported per μg of protein.

RESULTS

Figure 1 illustrates the time-course of PO_4 uptake by five placental BBM preparations at a substrate concentration of 0.04 mM, with and without 100 mM NaSCN in the incubation medium. For the first 30 s the uptake is linear and reaches a peak of 0.6 nmol, mg protein $^{-1}$, at 2 min. The PO_4 content of the vesicles subsequently decreases and reaches a relatively steady state between 15 and 30 min. When Na is replaced by K, no overshoot is observed, and at 2 min, the uptake is 6-fold lower than in the presence of a Na gradient. At 20 min, the PO_4 trapped in the vesicles is still 3-fold higher when Na is present than when it is absent. Not shown in Figure 1, the PO_4 uptake is greatly reduced by the addiiton of NaAs 1 mM in the incubation medium, and kinetic experiments using several concentrations of substrates show that As is a competitive inhibitor.

In the other figures, the data presented only concern the Na-dependent PO_4 uptake, *.ie.* the difference in PO_4 uptake in the presence and absence of the 100 mM Na gradient during the first 30 s of incubation.

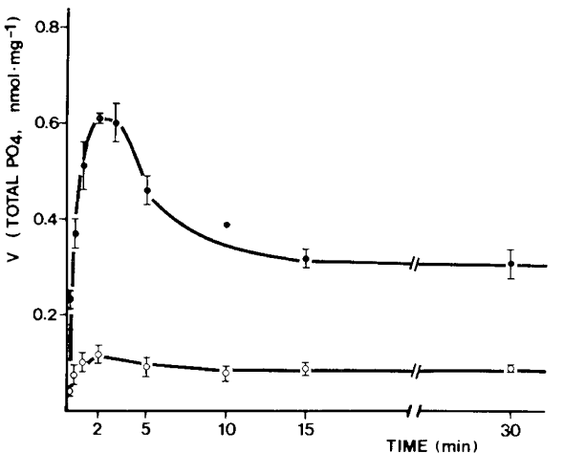

Fig. 1. Time-course of PO_4 uptake by isolated placental BBM vesicles: ● with 100 mM NaSCN in the incubation medium, ○ with 0 mM NaSCN but 100 mM KSCN in the incubation medium, at pH 7.5, T° 35° C. Substrate concentration: 0.045 mM.

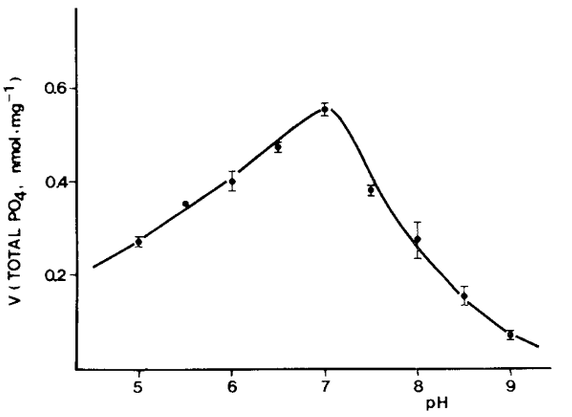

Fig. 2. Influence of pH on total PO_4 uptake by placental BBM vesicles, at substrate concentration 0.045 mM, T° 35° C.

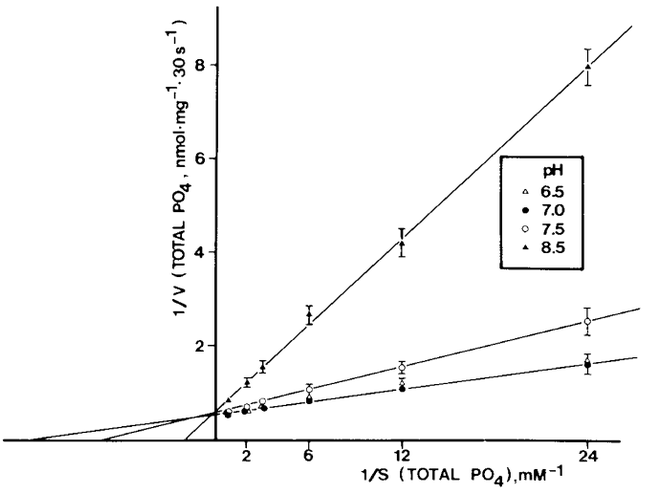

Fig. 3. Lineweaver-Burk plot of PO_4 uptake at varying pH, T° 35° C. The pH inside and outside the vesicles was the same. The total PO_4 (mono and divalent forms) was considered as the substrate.

Figure 2 illustrates the influence of pH on the PO_4 uptake. The substrate concentration in these studies was 0.04 mM. The pH was identical inside and outside the vesicles. Uptake is greatly modified by pH, the maximum being obtained with a pH slightly under 7.

Figure 3 shows the Lineweaver-Burk plot of total PO_4 uptake at four different pH. Whereas maximum uptake is obtained between 6.5 and 7 with a low substrate concentration, at high substrate concentration the influence of pH becomes negligible, so that V_{max} is identical at any pH, approximating 1.85 nmol mg^{-1} $30s^{-1}$ (Table 1).

In an attempt to clarify whether the pH influences PO_4 transport by 1) changing the transport system or 2) changing the proportion of monovalent *versus* divalent forms of the substrate or both, the data presented in Figure 3 were replotted, taking the diphosphate or the monophosphate as the single hypothetical substrate. Indeed, it was assumed that if only one form of substrate were transported, plotting this form as substrate would result in a unique Lineweaver-Burk plot, independent of pH. This was not the case. Instead of obtaining a single Lineweaver-Burk plot whatever the pH, a multiple set of regression lines was obtained, similar to those in Figure 3, with various Kms and one V_{max} (Tables 1 and 2).

Table 1. *Influence of pH on the apparent Km values when total PO_4, the divalent or monovalent forms of PO_4 are considered as the substrate**

	pH	Apparent K_m (μM) S = Tot PO_4	S = PO_4^{--}	S = PO_4^{-}	No. of experiments
(1)	6.5	84.6 ± 3.6	28.4 ± 1.1	57.4 ± 2.4	5
(2)	7.0	86.5 ± 24	54.5 ± 15	32.5 ± 9.5	2
(3)	7.5	153 ± 15	129 ± 13	25.4 ± 2.5	5
(4)	8.5	505 ± 55	505 ± 55	19.7 ± 3.0	3

Scheffe's multiple contrast (Zar JH 1974 Biostatistical Analysis. W. D. McElroy and C. P. Swanson (eds). p 155):

(1) *vs* (2)	NS	NS	NS
(1) *vs* (3)	NS	$p < 0.02$	NS
(1) *vs* (4)	$p < 0.001$	$p < 0.001$	$p < 0.001$
(2) *vs* (3)	NS	NS	NS
(2) *vs* (4)	$p < 0.001$	$p < 0.001$	$p < 0.001$
(3) *vs* (4)	$p < 0.001$	$p < 0.001$	$p < 0.001$

* The monovalent and divalent form of phosphate were calculated using a pK_2 corrected for ionic strength of 6.8 (Segel IH 1976 Biochemical Calculations. John Wiley and Sons Inc, New York, pp 66–67).

Table 2. *Influence of pH on the V_{max} of PO_4 uptake when the total PO_4, the divalent or monovalent forms of PO_4 are considered as the substrate*

pH	V_{max} (nmol mg^{-1} 30 s^{-1}) S = Tot PO_4	S = PO_4^{--}	S = PO_4^{-}	*n*
6.5	1.82 ± 0.18	1.82 ± 0.18	1.84 ± 0.18	5
7.0	1.90 ± 0.18	1.93 ± 0.20	1.89 ± 0.20	2
7.5	1.85 ± 0.08	1.85 ± 0.08	1.85 ± 0.08	5
8.5	1.69 ± 0.21	1.68 ± 0.21	2.64 ± 0.44	3

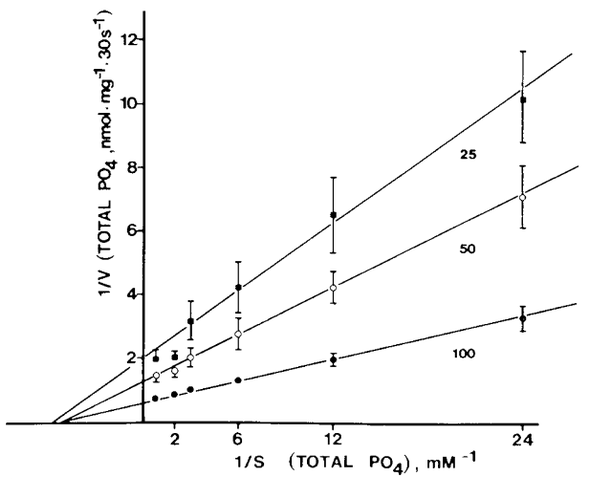

Fig. 4. Lineweaver-Burk plot of PO_4 uptake when various concentrations (25–50–100 mM) of Na were present in the incubation medium. No Na was present inside the vesicles. pH: 7.5, T° 35° C.

Table 3. *Influence of Na content in the incubating medium on Michaelis-Menton constants of PO_4 uptake at pH 7.5*

Na (mM)	App Km (μM)	V_{max} (nmol mg^{-1} 30 s^{-1})	*n*
25	211 ± 16	0.63 ± 0.13	4
50	199 ± 7	0.86 ± 0.13*	4
100	162 ± 11†	1.56 ± 0.17‡	4

Student's *t* test: * $p < 0.001$, † $p < 0.05$, ‡ $p < 0.005$ compared to the coresponding value with Na = 25 mM.

Figure 4 represents the influence of Na concentration on the kinetics of PO_4 uptake. Increasing the Na concentraton accelerated the initial PO_4 transport, but did not change the affinity of the transporting system for the substrate (Table 3).

DISCUSSION

Stulc and coworkers (7, 8) recently reported that the maternal-fetal transfer of PO_4 is an active phenomenon which decreases with inhibition of placental metabolism and is highly dependent upon the concentration of Na in the perfusate. Our experiments confirm the existence of a sodium cotransport system for the active PO_4 transport through the placental cell, and localize this particular Na-dependent transport at the BBM facing the maternal blood compartment. The overshoot phenomenon, already reported for PO_4 transport through renal and intestinal BBM, is interpreted as being the result of a rapid initial uptake energized by the Na gradient; following gradient dissipation, part of the PO_4 trapped in the vesicles backflows to the medium, to reach very slowly an equilibrium of the "inward" and "outward" movements of the electrolyte. As observed with the kidney vesicles (13, 15, 16) the intravesicular PO_4 remains higher in presence than in absence of sodium in the medium for 1 to 2 h. Since at that time, the sodium gradient is already dissipated, the maintenance of a higher PO_4 concentration in the vesicles than in the incubation medium, further suggests an active sodium–PO_4 cotransport mechanism. After 2 h (not shown on the figure) this PO_4 gradient has disappeared.

Based on the steady state values of PO_4 uptake by the vesicles after a 2-h incubation, the intravesicular volume is 2.5 μl/mg protein, a value which is very close to that reported for kidney BBM by Hoffman *et al.* (13) (4.5 μl/mg protein) and identical to that reported by us, also for renal BBM: 2.5 μl/mg protein (17).

PO_4 uptake by the placental BBM at 35° C is relatively slow compared to the kidney BBM at the same termperature: the initial velocity is lower in human placenta (1.84 ± 0.08 nmol mg^{-1} 30 s^{-1}) than in the rat kidney (3.56 ± 0.28 nmol mg^{-1} 10 s^{-1}) and the peak of uptake is reached later in the first membrane preparation than in the second one. Unfortunately, comparison with human kidney BBM is not possible since no data are available concerning PO_4 transport at 35° C. It is also not known whether this slow transport process is generalized for placental BBM or not, since studies concerning other substrates, particularly amino acids (18) and monosaccharids (19) dealt with equilibrium exchanges rather than initial uptakes.

As for the kidney, the Km value of placental BBM for PO_4 is far below the presumed concentration in maternal blood. PO_4 transport *in vivo*, consequently, seems to operate under saturation conditions, and does not depend upon the maternal plasma PO_4 concentrations.

The comparison of the effect of pH on PO_4 transport through BBM prepared from placenta, kidney, and intestine is complex because of the variety of experimental conditions used in these studies. Hoffman *et al.* (13) reported that PO_4 uptake by renal BBM increases with alkalinity. The range of pH studied was 6.0 to 8.0. They concluded that the divalent form of PO_4 was preferentially transported. Sacktor and Cheng (20) in the absence of a sodium gradient reported that acidity inside the vesicles enhances PO_4 uptake when the pH outside is kept constant at 7.4. In contrast, in a sodium gradient situation (21), increasing the pH inside and outside the vesicles from 5.6 to 8, while keeping the divalent phosphate constant, increases PO_4 uptake by 50%. They concluded that alkaline pH increases PO_4 transport not only by augmenting the divalent form, but also by stimulating the transport system itself. Recently, our laboratory (15) confirmed that the divalent form of PO_4 is preferentially transported, and that pH influences the carrier systerm by changing only the V_{max}, the optimal pH being 7.5. Indeed, when the divalent form was considered as the unique substrate, the pH did not modify the Km value. In intestine, Danisi *et al.* (22) confirm-

ing the previous findings of Kinne *et al.* (15) also demonstrated a pH dependency of PO_4 transport through the BBM. In contrast to what we reported for kidney BBM, reducing the pH from 8.1 to 5.7 doubles the rate of PO_4 influx by increasing the V_{max}. When calculated separately for each form (monovalent and divalent) of PO_4 the Km was also influenced by pH. They concluded that in intestine, both forms are transported, and that the pH dependence was in part due to changes in the affinity of the transporting system.

In the placenta, we also observed a striking influence of pH on PO_4 uptake with maximal values at pH slightly under 7 which corresponds to the pK of the PO_4 anions. This finding suggests that, in contrast to renal BBM but as found with intestinal BBM, the placental BBM transports the two forms of PO_4 indiscriminately and that the sensitivity of the transport system to pH is due to alterations in the transport system itself rather than to changes in the ratio of monovalent to divalent phosphate. The failure to obtain a single Lineweaver-Burk line, with constant V_{max} and Km at any pH when either PO_4^- or PO_4^{--} are plotted as the substrate, further confirms that pH influences the affinity of the carrier, whatever the species of PO_4 transported, the monovalent or divalent form. The mechanism of this influence, however, is not known.

Very few studies have been concerned with the manner in which Na increases PO_4 transport through the BBM from intestine and kidney. Danisi *et al.* (22) studied the kinetic constants of sodium dependent phosphate influx with two sodium concentrations (40 and 100 mM). No change in Km was observed. In kidney BBM, we recently reported that an increase in sodium was accompanied by a dramatic enhancement of the affinity of the carrier for PO_4, with only a modest change in the V_{max} (23).

The reverse is observed in the placenta. Such differences reflect the diversity of the systems responsible for PO_4 transport through the various biological membranes: whereas some are energized by proton gradients such as in mitochondria (24, 25), others require a Na gradient. In the latter group, the Na gradient may affect PO_4 uptake by changing the affinity as in the kidney, or the V_{max} as we have shown for the placenta.

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