# Mechanisms of intestinal phosphate transport in small ruminants

BY B. SCHRÖDER[1], H. KÄPPNER[1], K. FAILING[1], E. PFEFFER[2]
AND G. BREVES[1]

[1] *Department of Veterinary Physiology, Justus-Liebig-University, Giessen, Germany*
[2] *Department of Animal Nutrition, Rheinische Friedrich-Wilhelms-University, Bonn, Germany*

In order to study the localization and mechanisms of intestinal phosphate transport in sheep and goats, unidirectional inorganic phosphate ($P_i$) flux rates across isolated stripped epithelial tissues were measured *in vitro* by applying the Ussing-chamber technique. In the first experiment the tissues were obtained from animals which had been kept on an adequate dietary P supply. In the second experiment the animals had either been kept on an adequate Ca and P supply or were Ca- and/or P-depleted. Significant net $P_i$ absorption was measured in all segments of the small intestine and in the proximal colon of sheep and in the duodenum and jejunum of goats. Since the experiments were carried out in the absence of any electrochemical gradient, this clearly indicates the presence of active mechanisms for $P_i$ transport in the intestinal tract of small ruminants. In sheep jejunum, reduction of mucosal Na concentration to 1·8 mM or serosal application of ouabain (0·1 mM) resulted in significant decreases of net $P_i$ absorption of the same order of magnitude, indicating that about 65 % of active $P_i$ transport in sheep jejunum is mediated by a Na-dependent active transport mechanism. The mechanism for the remaining $Na^+$-independent active $P_i$ transport has not yet been identified. Dietary P depletion caused hypophosphataemia and induced a significant stimulation of net $P_i$ absorption in goat duodenum and jejunum. This increase was independent of dietary Ca supply and was not associated with increased plasma calcitriol concentrations. This suggests substantial differences in hormonal regulation of $P_i$ transport in small ruminants in comparison with single-stomached species.

Intestinal phosphate absorption: Phosphorus: Calcium: Ruminant

In ruminants large amounts of inorganic phosphate ($P_i$) enter the gastrointestinal tract by salivary secretion, and this endogenous secretion is well balanced by net $P_i$ absorption from the intestinal tract. Thus the overall absorption rate from the intestinal tract is substantially higher compared with single-stomached animals (for review see Breves & Schröder, 1991). Although it is well known that in ruminants the upper small intestine is the major site for net $P_i$ absorption, little is known about the epithelial transport processes involved and their hormonal regulation. This is in contrast to many single-stomached species where a calcitriol-stimulated secondary active $Na^+$–$P_i$-cotransport system has been identified as the predominant mechanism for active $P_i$ absorption (Bruce *et al.* 1966/67; Pfeffer *et al.* 1970; Grace *et al.* 1974; Berner *et al.* 1976; Murer & Hildmann, 1981; Brandis *et al.* 1987; Danisi *et al.* 1988; Shirazi-Beechey *et al.* 1988).

For sheep a carrier-mediated mechanism has been assumed for mucosal $P_i$ uptake from *in vivo* studies (Care *et al.* 1980; Scott *et al.* 1984). From a recent *in vitro* study with isolated brush border membrane vesicles from sheep, evidence was obtained for active $P_i$ uptake, which was driven by a transmembrane proton gradient and which was stimulated in response to dietary P depletion (Shirazi-Beechey *et al.* 1991). However, it has to be excluded that this adaptation was controlled by changes in plasma calcitriol since it is well known from different studies that in ruminants neither calcitriol nor its production rate is changed

in response to dietary P depletion as has been described for single-stomached species (Breves *et al.* 1985; Maunder *et al.* 1986; Schröder *et al.* 1990).

In order to get more detailed knowledge about intestinal $P_i$ transport mechanisms in sheep and goats, Ussing chambers were used for studying transepithelial $P_i$ flux rates in the absence of electrochemical gradients. In the first experiment the intestinal tissues were obtained from animals which had been kept on an adequate P supply. In the second experiment the tissues were taken from growing goats which had either been kept on an adequate dietary Ca and P supply or were Ca- and/or P-depleted.

## MATERIALS AND METHODS
### Animals and feeding

In Expt 1, intestinal tissues were obtained from adult sheep and goats weighing between 10 and 65 kg, which had been kept on an adequate dietary Ca and P supply. In Expt 2 the tissues were taken from male growing goats of 4–5 months of age. Nine weeks before slaughter animals were allotted to four different dietary regimens with either adequate or reduced Ca and/or P contents (Table 1). From all animals of Expt 2, blood samples were taken the day before slaughter and plasma was stored at $-70°$. Intestinal segments were taken within 3 min after exsanguination, from either the descending part of the duodenum, mid jejunum, terminal ileum or proximal colon. They were immediately rinsed with ice-cold physiological saline (9 g NaCl/l) and kept in a glucose-containing buffer solution at $4°$, being continuously gassed with $O_2$–$CO_2$ (95:5 v/v).

### Incubation of epithelial tissues and calculation of $P_i$ flux rates

In each experiment intestinal segments were opened along the mesenteric line and fixed on a Pyrex plate with the mucosal surface uppermost. Epithelial tissues were stripped off the muscle layers and mounted in computer-controlled Ussing chambers (Mussler Microclamp, Aachen, Germany) with an exposed area of 100 mm². The tissues were incubated on both sides with 13 ml of an isotonic buffer solution (pH 7·4) containing (mmol/l) NaCl 125·4, KCl 5·4, $CaCl_2$ 1·2, $NaHCO_3$ 21, $Na_2HPO_4$ 0·3, $NaH_2PO_4$ 1·2 and indomethacin 0·01. In addition, the serosal solution contained 10 mM glucose, which for osmotic reasons was compensated on the mucosal side by 10 mM-mannitol. In some experiments choline and K salts were used to reduce Na concentrations from 148·2 mM to 1·8 mM. In order to check the functional viability of the tissues at the start and the end of flux measurements maximal increases of short-circuit currents ($I_{sc}$) were determined in a number of experiments in response to either theophylline (10 mM serosal) or glucose (10 mM mucosal) or L-alanine (5 mM mucosal).

For calculations of unidirectional $P_i$ flux rates ($J_{ms}$: flux from mucosal to serosal side, $J_{sm}$: flux in the opposite direction), 185 KBq [³²P]orthophosphate (370 MBq/ml, Amersham Buchler, Braunschweig, Germany) were added to one side of the tissue. Samples (0·1 ml) were taken from the labelled side 20 min later and subsequently in at least three 10 min intervals from the unlabelled side (0·5 ml) of the Ussing chamber. All samples taken from the unlabelled side were replaced by equal volumes of isosmotic bathing fluid. Net fluxes ($J_{net}$) were calculated as differences between $J_{ms}$ and $J_{sm}$ of paired tissues whose conductances did not differ by more than 25%. In another series of experiments flux measurements were done before and after the addition of arsenate (mucosal) or ouabain (serosal) to the bathing solution. Radioactivity measurements were done in a Packard Tricarb liquid scintillation counter with counting accuracies > 95%. For further experimental details see Schröder *et al.* (1991, 1993*a*). Unidirectional $P_i$-flux rates were calculated using standard equations (Schultz & Zalusky, 1964).

Table 1. *Dietary calcium and phosphorus contents of rations fed to growing goats*

| Grouping* | | Feed (g/kg dry matter) | |
| --- | --- | --- | --- |
| Ca | P | Ca | P |
| + | + | 11·1 | 4·6 |
| + | − | 10·1 | 2·0 |
| − | + | 4·2 | 4·5 |
| − | − | 3·9 | 1·9 |

+, adequate mineral content; −, reduced mineral content.

### Analytical procedures

Plasma Ca and $P_i$ were measured using test kits provided by Boehringer (Mannheim, Germany).

Plasma parathyroid hormone (PTH) was determined using a commercial PTH assay system, which detects the midregional 44–68 amino acid sequence fragment of the entire human PTH polypeptide (Immunodiagnostik, Bensheim, Germany). This assay system was used due to lack of appropriate kits to measure intact PTH or PTH fragments in goat plasma. The detection limit for measuring the hPTH(44–68) immunoreactive fraction in goat plasma was < 16 PM. Calcitriol concentrations in goat plasma were measured according to the method of Hollis (1986) involving single cartridge extraction and separation of calcitriol from plasma (Bond Elut C18-OH; ICT, Bad Homburg, Germany) and subsequent determination of calcitriol using a competitive protein-binding assay with a preparation of calf thymus cytosol (Reinhardt & Horst, 1990). To assess sufficient analytical recovery of calcitriol the extraction and separation procedure was modified as follows: to 2 ml goat plasma 10 $\mu$l [³H]calcitriol in ethanol (67 Bq, specific activity 6·36 TBq/mmol; Amersham Buchler) was added. After vortex-mixing the sample was incubated for 60 min at room temperature. Acetonitrile extraction of plasma vitamin D metabolites from the sample was performed twice for 30 min each on a shaker. After centrifugation and dilution of combined supernatant fractions with 1 volume of 0·4 mM-$K_2HPO_4$ (pH 10·5) the entire extract was applied to a prewashed C18-OH cartridge (2 ml hexane, 2 ml isopropanol, 2 ml water). Separation of calitriol from the plasma extract was performed by washing the cartridge successively with 5 ml water, 5 ml methanol–water (70:30 v/v), 5 ml hexane–methylene chloride (90:10 v/v), 5 ml hexane–isopropanol (99:1 v/v), and finally elution of the calcitriol-containing fraction with 6 ml hexane–isopropanol (94:6 v/v). All solvent elutions of the cartridges were performed under reduced pressure using an Adsorbex sample preparation unit (Merck, Darmstadt, Germany). Analytical recovery of [³H]calcitriol through the entire extraction and purification procedure was 48·1 (SD 15·9)%, (*n* 32). The sensitivity of the nonequilibrium competitive receptor binding assay using the specific vitamin D receptor from calf thymus, defined as 2 SD from the mean for data from the zero sample, was 2 pg/tube. Bound tracer was 50% displaced at approximately 10 pg/tube. In the absence of calcitriol standard, approximately 25% of the total [³H]calcitriol was bound to the thymus receptor.

### Other chemicals

The solvents were purchased from Merck (Darmstadt, Germany). The chemicals for preparing the buffer solutions, arsenate, ouabain, theophylline and L-alanine were obtained from Sigma Chemie GmbH (Deisenhofen, Germany). All chemicals were of analytical

grade. Unlabelled calcitriol was kindly provided by Hoffman-La Roche (Basel, Switzerland).

## Statistics

All calculations were performed using the BMDP-87 software (Dixon, 1987). Univariate statistics such as arithmetic mean, standard deviation (SD) and standard error of the mean (SE) of the appropriate number of animals were computed for each variable by the BMDP1D program. In general, $P$ values $< 0.05$ were taken to be statistically significant. According to the design of the experiments assessment of consecutive experimental periods in a given preparation (same animal, same segment) were based on paired statistics. In contrast, comparisons of preparations from different localizations were based on unpaired statistics because in most of the experiments the individual intestinal segments were derived from different animals. Paired and unpaired Student's $t$ tests were used for statistical analysis (BMDP3D). One-way and two-way ANOVA were performed using the BMDP2V routine. For a significant result, individual mean values were checked in pairs for significant differences using the conventional Scheffé test (Sachs, 1992).

## RESULTS

### Experiment 1

*Viability and electrical properties of intestinal epithelia from goats and sheep*. In both species no significant changes of short-circuit currents ($I_{sc}$) and tissue conductances ($G_T$) occurred during the experimental period of at least 2 h, indicating constant conditions during the experiments. In addition, in a number of experiments tissues were either treated with theophylline (sheep duodenum) or glucose (duodenum of growing goats) or L-alanine (jejunum of growing goats) at the end of the flux measurements in order to check tissue viability (Fig. 1). Theophylline was used to increase second messenger concentrations, i.e. cyclic nucleotides, in the enterocytes. Glucose and L-alanine were both used in order to increase electrogenic $Na^+$ transport which resulted in increase of $I_{sc}$. In response to these compounds no significant differences in maximal increases of $I_{sc}$ were detected in comparison with respective initial treatments after mounting the tissues (results not shown).

In the duodenum of adult goats $I_{sc}$ values were positive and significantly higher ($P < 0.001$, paired $t$ test) compared with those for jejunal preparations (Table 2). The negative jejunal $I_{sc}$ may have resulted from either net absorption of anions or net secretion of cations. $G_T$ values in goat duodenum were significantly lower than in the jejunum and in all intestinal segments of sheep. In sheep, $G_T$ decreased from the duodenum to the proximal colon. In sheep all $I_{sc}$ were positive and significantly higher in the ileum and proximal colon compared with the upper parts of the small intestines (Table 2).

*Intestinal $P_i$ flux rates in goats and sheep*. In both species and in all segments of the intestinal tract $J_{ms}$ exceeded $J_{sm}$ under short-circuit-current conditions resulting in significant net flux rates from the mucosal to the serosal compartment (Table 3). $J_{net}$ values in goat jejunum were significantly higher than in the duodenum. In sheep, $J_{net}$ slightly increased within the distal parts of the small intestines.

*Effects of arsenate, sodium substitution and ouabain on jejunal net $P_i$ flux rates in sheep*. When arsenate was added at concentrations of 2, 5 or 7 mM to the mucosal bathing solution, $J_{net}$ of $P_i$ decreased. Maximal inhibition of about 65 % was achieved by 5 and 7 mM-arsenate (Fig. 2). A similar inhibition of $J_{net}$ was observed either when mucosal Na was reduced from 148.2 mM to 1.8 mM (Fig. 3) or after serosal application of 0.1 mM-
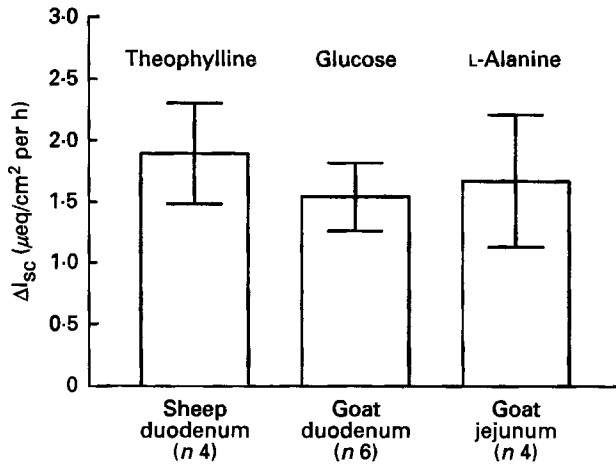
Fig. 1. Maximal increases of short-circuit current ($\Delta I_{sc}$) across small-intestinal epithelia from sheep and growing goats, in response to different substances. Theophylline (10 mM in serosal bathing solution), glucose (10 mM in mucosal fluid) and L-alanine (5 mM in mucosal fluid) were added at the end of unidirectional flux measurements. For osmotic reasons equimolar amounts of mannitol were given to the untreated compartment. Values are means with their standard errors represented by vertical bars. For details of procedures, see p. 636.

Table 2. *Electrical properties of stripped epithelia from goats and sheep*

(Mean values with their standard errors)

| Species | Segment | $n$ | $I_{sc}$ ($\mu$eq/cm$^2$ per h) | | $G_T$ (mS/cm$^2$) | |
|---|---|---|---|---|---|---|
| | | | Mean | SE | Mean | SE |
| Goat | Duodenum | 13 | 0·41 | 0·05 | 13·5 | 1·1 |
| | Jejunum | 13 | −1·04 | 0·29 | 28·1 | 1·2 |
| Sheep | Duodenum | 9 | 0·45 | 0·07 | 30·3 | 3·4 |
| | Jejunum | 30 | 0·29 | 0·11 | 26·4 | 1·5 |
| | Ileum | 12 | 1·93 | 0·42 | 26·6 | 2·9 |
| | Proximal colon | 6 | 1·55 | 0·26 | 22·0 | 2·1 |

$I_{sc}$, short-circuit current; $G_T$, tissue conductance.

ouabain (Fig. 4). In each of these experiments remaining $J_{net}$ values were still significantly different from zero.

*Experiment 2*

*Variables of calcium and phosphorus homeostasis in goats.* P depletion resulted in hypophosphataemia and hypercalcaemia and these changes were not related to the level of Ca intake (Table 4). In Ca-depleted goats no significant changes in plasma Ca concentrations could be detected. These animals, however, became hyperphosphataemic when they were kept on an adequate P supply. In both Ca-depleted groups calcitriol concentrations significantly increased in comparison with control animals. Plasma PTH levels were only higher in Ca depletion without P depletion as indicated by a significant interaction between dietary Ca and P conditions (Table 4).

*$P_i$ flux rates and electrical properties of duodenal and jejunal tissues as affected by calcium and/or phosphorus depletion.* In both duodenal and jejunal tissues $J_{ms}$ increased significantly with low P intake irrespective of the level of Ca intake. Since $J_{sm}$ remained unaffected this

Table 3. *Unidirectional and net flux rate of inorganic phosphate in intestinal segments from goats and sheep* *

(Mean values with their standard errors)

| Species | Segment | $n$ | $J_{ms}$ (nmol/cm² per h) | | $J_{sm}$ (nmol/cm² per h) | | $J_{net}$ (nmol/cm² per h) | | $P$ |
|---------|---------|-----|------|-----|------|-----|------|-----|-----|
| | | | Mean | SE | Mean | SE | Mean | SE | |
| Goat | Duodenum | 13 | 33·9 | 4·2 | 12·4 | 2·3 | 21·5 | 2·8 | < 0·001 |
| | Jejunum | 13 | 114·8 | 14·7 | 40·7 | 11·3 | 74·1 | 15·0 | < 0·001 |
| Sheep | Duodenum | 9 | 69·1 | 12·0 | 43·2 | 6·7 | 25·9 | 6·8 | < 0·01 |
| | Jejunum | 30 | 87·7 | 7·5 | 34·0 | 3·3 | 53·8 | 7·2 | < 0·001 |
| | Ileum | 12 | 131·1 | 17·1 | 42·8 | 7·2 | 88·2 | 19·2 | < 0·001 |
| | Proximal colon | 6 | 52·6 | 4·5 | 29·3 | 3·8 | 23·3 | 6·8 | < 0·05 |

$J_{ms}$, mucosal to serosal flux rate; $J_{sm}$, serosal to mucosal flux rate; $J_{net}$, net flux rate ($J_{net} = J_{ms} - J_{sm}$); $P$, statistical significance of difference from zero of mean $J_{net}$.

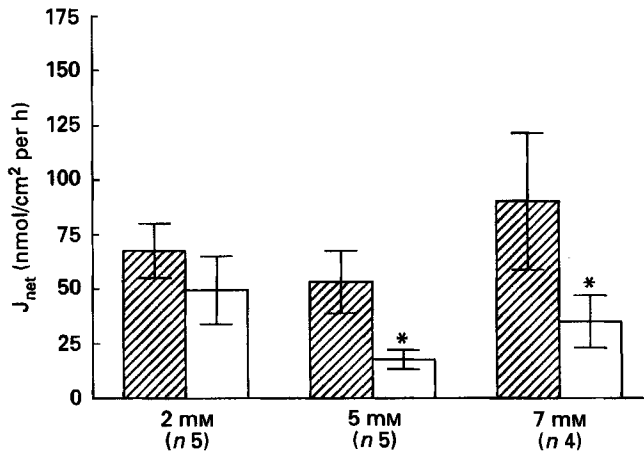* For details of procedures, see p. 636.



Fig. 2. Inhibition of net flux rates ($J_{net}$) of inorganic phosphate across jejunal epithelia from sheep in the presence of mucosal arsenate. Values are means with their standard errors represented by vertical bars; (▨), basal; (□), arsenate-treated. Values for inhibition as a percentage of basal $J_{net}$ were: 2 mM −27·0, 5 mM −66·8, 7 mM −61·2. *Mean values were significantly different from control, $P < 0·05$. For details of procedures, see p. 636.

change resulted in significant increases of $J_{net}$ in both intestinal segments (Tables 5 and 6). Electrical properties of duodenal tissues were not affected by dietary treatment whereas in jejunal tissues P depletion induced increases in negative $I_{sc}$ compared with both adequately P-fed groups (Tables 5 and 6). In all these experiments no statistically significant interactions could be observed.

DISCUSSION

*Viability of the tissues during experiments*

*In vitro* transepithelial flux measurements across intact intestinal epithelia with the Ussing-chamber system require sufficient integrity and viability of the tissues during the experimental period. Functional viability of the epithelia over at least 2 h has been
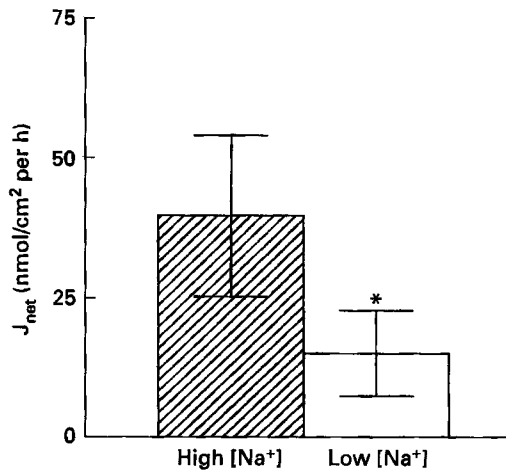
Fig. 3. Change in the net flux rate ($J_{net}$) of inorganic phosphate across jejunal epithelia from sheep in response to reducing the sodium concentration in the mucosal bathing solution from 144 mM (high [$Na^+$]) to 1·8 mM (low [$Na^+$]). Values are means with their standard errors represented by vertical bars for four animals for four experiments. Inhibition as a percentage of basal $J_{net}$ was $-61·9$. * Mean value was significantly different from high [$Na^+$], $P < 0·05$. For details of procedures, see p. 636.
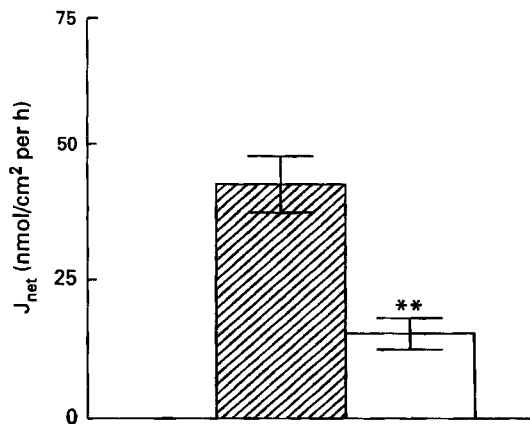
Fig. 4. Inhibition of net flux rates ($J_{net}$) of inorganic phosphate aross jejunal epithelia from sheep in the presence of serosal ouabain (0·1 mM). Values are means with their standard errors represented by vertical bars, for six experiments for six animals; (▨), basal; (□), ouabain-treated. Inhibition as a percentage of basal $J_{net}$ was $-63·3$. ** Mean value was significantly different from basal, $P < 0·01$. For details of procedures, see p. 636.

demonstrated by measuring short-circuit currents (electrogenic net ion transport) and tissue conductances (representing trans- and paracellular pathways). Neither electrical variable changed significantly during the experiments. Furthermore, addition of organic substrates such as glucose and L-alanine to the tissues or treatment with the secretagogue theophylline provoked similar increases of $I_{sc}$ at the start and the end of the experiments. Since the experiments were performed in the absence of any organic substrates in the luminal compartment, electrogenic $Na^+$/substrate-cotransport was kept at a low level

Table 4. *Concentrations of calcium, inorganic phosphate, parathyroid hormone, and calcitriol in plasma from growing goats as affected by long-term dietary calcium and/or phosphorus depletion*\*

(Mean values with their standard errors for eight animals per dietary group)

| Grouping | | Ca (mmol/l) | | $P_i$ (mmol/l) | | PTH (pmol/l) | | Calcitriol (pmol/l) | |
|---|---|---|---|---|---|---|---|---|---|
| Ca | P | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| + | + | 2·73 | 0·20 | 2·22 | 0·40 | 95 | 8 | 107 | 17 |
| + | − | 3·02 | 0·13 | 0·61 | 0·17 | 85 | 9 | 102 | 19 |
| − | + | 2·70 | 0·19 | 2·78 | 0·20 | 148 | 18 | 233 | 26 |
| − | − | 2·95 | 0·18 | 0·87 | 0·50 | 99 | 4 | 224 | 25 |
| Source of variation | | | | $P$ values of two-way ANOVA | | | | | |
| Ca | | NS | | < 0·01 | | < 0·01 | | < 0·001 | |
| P | | < 0·001 | | < 0·001 | | < 0·01 | | NS | |
| Ca × P interaction | | NS | | NS | | < 0·05 | | NS | |

$P_i$, inorganic phosphate; PTH, parathyroid hormone.
\* For details of treatments, see Table 1.

Table 5. *Unidirectional and net flux rates of inorganic phosphate, and electrical properties of duodenal preparations of growing goats as affected by calcium and/or phosphorus depletion*\*

(Mean values with their standard errors)

| Grouping | | | $J_{ms}$ (nmol/ cm² per h) | | $J_{sm}$ (nmol/ cm² per h) | | $J_{net}$ (nmol/ cm² per h) | | $I_{sc}$ ($\mu$eq/ cm² per h) | | $G_T$ (mS/cm²) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ca | P | n | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| + | + | 8 | 44·2 | 12·8 | 8·7 | 2·3 | 35·5 | 11·9 | 0·51 | 0·07 | 12·4 | 1·2 |
| + | − | 7 | 95·4 | 36·2 | 10·1 | 2·8 | 85·4 | 33·4 | 0·52 | 0·08 | 14·3 | 1·8 |
| − | + | 8 | 34·7 | 7·9 | 7·7 | 1·5 | 27·0 | 7·1 | 0·50 | 0·05 | 13·2 | 1·3 |
| − | − | 7 | 86·5 | 18·6 | 11·3 | 1·7 | 75·3 | 18·2 | 0·73 | 0·03 | 14·8 | 1·2 |
| Source of variation | | | | | | | $P$ values of two-way ANOVA | | | | | |
| Ca | | | NS | | NS | | NS | | NS | | NS | |
| P | | | < 0·05 | | NS | | < 0·05 | | NS | | NS | |
| Ca × P interaction | | | NS | | NS | | NS | | NS | | NS | |

$J_{ms}$, mucosal to serosal flux rate; $J_{sm}$, serosal to mucosal flux rate; $J_{net}$, net flux rate ($J_{net} = J_{ms} - J_{sm}$); $I_{sc}$, short-circuit current; $G_T$, tissue conductance.
\* For details of treatments, see Table 1.

during the experiments. This procedure gave the opportunity to test $Na^+$/substrate-cotransport capacity of the epithelia at the end of the flux measurements as indicated by increases of the $I_{sc}$ after addition of respective substrates (Ferrante *et al.* 1988). In order to prove the tissue's capability to stimulate electrolyte transport by second messengers, theophylline was added to the serosal buffer solution. This secretagogue is known to increase cytosolic cAMP and cGMP concentrations and intracellular free Ca levels (Schröder *et al.* 1991). Tissue viability and integrity could be confirmed by these functional

Table 6. *Unidirectional and net flux rates of inorganic phosphate, and electrical properties of jejunal preparations of growing goats as affected by calcium and/or phosphorus depletion\**

(Mean values with their standard errors)

| Grouping | | | $J_{ms}$ (nmol/ cm² per h) | | $J_{sm}$ (nmol/ cm² per h) | | $J_{net}$ (nmol/ cm² per h) | | $I_{sc}$ (μeq/ cm² per h) | | $G_T$ (mS/cm²) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ca | P | n | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| + | + | 8 | 86·1 | 12·2 | 34·5 | 3·2 | 51·6 | 11·3 | −0·81 | 0·23 | 27·8 | 2·0 |
| + | − | 7 | 171·6 | 38·1 | 39·0 | 6·6 | 132·6 | 36·2 | −1·35 | 0·24 | 28·9 | 1·4 |
| − | + | 8 | 122·2 | 26·5 | 30·8 | 5·8 | 91·5 | 24·7 | −0·31 | 0·25 | 26·1 | 2·8 |
| − | − | 7 | 156·2 | 31·9 | 32·1 | 5·9 | 124·1 | 32·6 | −1·01 | 0·44 | 29·5 | 1·7 |
| Source of variation | | | | | | | *P* values of two-way ANOVA | | | | | |
| Ca | | | NS | | NS | | NS | | NS | | NS | |
| P | | | < 0·05 | | NS | | < 0·05 | | < 0·05 | | NS | |
| Ca × P interaction | | | NS | | NS | | NS | | NS | | NS | |

$J_{ms}$, mucosal to serosal flux rate; $J_{sm}$, serosal to mucosal flux rate; $J_{net}$, net flux rate ($J_{net} = J_{ms} - J_{sm}$); $I_{sc}$, short-circuit current; $G_T$, tissue conductance.

\* For details of treatments, see Table 1.

tests since the epithelial response was identical at both start and end of the flux measurements. It could further be confirmed by the constancy of the successive $P_i$ flux rates in individual Ussing chambers.

### Active $P_i$ absorption in the small intestine and the proximal colon

In both goats and sheep, $J_{ms}$ exceeded $J_{sm}$ in all intestinal segments tested in the present study, thus resulting in significant net $P_i$ absorption (Table 3). These $J_{net}$ clearly indicate the presence of active mechanisms for $P_i$ transport in the small intestine and the proximal colon since they were measured in the absence of any transepithelial electrochemical gradients. These findings are the first experimental proof for actively driven $P_i$ transport obtained from intact intestinal epithelia of ruminants, thus confirming the assumptions made by Care *et al.* (1980), Scott *et al.* (1984) and Scharrer (1985) who have postulated the involvement of carrier-mediated mechanisms in the intestinal transport of $P_i$ in sheep. In the small intestine, $J_{net}$ of $P_i$ increased from oral to aboral. The reasons for this are not yet fully understood. Physiological as well as experimental conditions may be involved. Different $P_i$ transport capacities along the intestinal tract, i.e. different $V_{max}$ values of the brush order $Na^+/P_i$ cotransport system, can cause different flux rates in respective intestinal segments as has been described for pigs and rabbits (Brandis *et al.* 1987; Danisi & Murer, 1991).

In the present study the physiological pH value of the extracellular fluid (7·4) was adjusted in both mucosal and serosal solutions. Thus, it has to be taken into account that a mucosal pH of 7·4 was only equivalent to *in vivo* conditions using tissues from the mid jejunum. From experiments with sheep a pH range of 3·5 to 5·2 has been reported for the luminal fluid in the upper small intestine (1–3 m from the pylorus) and 7·8 to 8·2 in the terminal ileum (Ben-Ghedalia *et al.* 1975). However, the main principle to detect active transport mechanisms with the Ussing-chamber technique implies the absence of any electrochemical gradient which also excludes the application of transepithelial pH gradients. Therefore, the

present study was performed under iso-pH conditions. Nevertheless, it still has to be proved for ruminants how luminal pH reflects cell surface pH. It has been shown for rats and pigs that the mucosal surface is isolated from changes in the pH of luminal contents by a zone of moderate pH ('acid microclimate') (Daniel *et al.* 1989; McEwan *et al.* 1990). In conclusion, it cannot be excluded for duodenal tissues that factors such as the ratio between mono- and divalent $P_i$ and proton gradients present under *in vivo* conditions may have been underestimated.

*Sodium dependency of $P_i$ transport*

Since it is well established that in ruminants, as in single-stomached species, the small intestine is the major site for net $P_i$ absorption, and with respect to physiological pH conditions, tissues from the mid jejunum were used for further characterizing the properties of active $P_i$ transport in sheep. It could be demonstrated that almost 0·66 of active $P_i$ transport depended on the presence of Na since the reduction of mucosal Na concentration from 148·2 mM to 1·8 mM reduced $J_{net}$ by about 60% (Fig. 3). Similar findings were obtained when ouabain was added to the serosal compartment at a concentration of 0·1 mM (Fig. 4). This concentration had been found to be sufficient for complete inhibition of basolateral $Na^+$-$K^+$-ATPase (*EC* 3.6.1.3) activity (Blaustein, 1993). In further studies arsenate was added to the mucosal side of the intact tissues resulting in a significant decrease of $J_{net}$ of about 65% in comparison with controls. Maximal inhibition was found at a concentration of 5 mM. Adjusting arsenate to 7 mM did not further increase the degree of inhibition (Fig. 2). Arsenate, an anion analogous in structure to the phosphate anion, has been used as a competitive inhibitor of the renal and intestinal $Na^+$-coupled $P_i$ cotransport system in *in vitro* $P_i$ uptake measurements with isolated brush border membrane vesicles (BBMV) from single-stomached species (Berner *et al.* 1976; Hoffmann *et al.* 1976; Danisi *et al.* 1984; Quamme, 1985; Shirazi-Beechey *et al.* 1988). This inhibitory effect has been confirmed by recent studies with intestinal BBMV from goats where $Na^+$-dependent $P_i$ uptake could be inhibited completely in the presence of arsenate (Schröder *et al.* 1993b). However, the fact that the level of inhibition by arsenate was similar to the effects in response to the reduction of Na in the mucosal buffer solution, or in response to addition of ouabain in the serosal compartment, may be a coincidence, since arsenate may also have a variety of other effects on living cells, i.e. intermediary metabolism, which can limit the suitability of this compound for studies of active $P_i$ transport system (Szczepanska-Konkel *et al.* 1986).

Although from the inhibition and substitution experiments the Na-dependency of active intestinal $P_i$ transport in sheep has been shown, it may not be concluded that this mechanism is identical with the secondary active $Na^+/P_i$ cotransport which has been shown in different single-stomached species (for review see Breves & Schröder, 1991) and sheep ileum (Schneider *et al.* 1985a, b). The observed effect of $Na^+$ could also be due to the functioning of a $Na^+/H^+$ antiporter which may be present in the luminal membrane. $Na^+$-driven $H^+$ extrusion could provide protons which could be used to be coupled to the movement of $P_i$ into the cell via a $H^+/P_i$ cotransporter as has been shown for sheep duodenum (Shirazi-Beechey *et al.* 1989, 1991). A similar mechanism has also been demonstrated for the internal mitochondrial membrane of eukaryotic cells (Wehrle & Pedersen, 1989). Hence the exclusion of $Na^+$ from the luminal compartment would lead to the reduction of activity of the $Na^+/H^+$ antiporter and this could consequently reduce the rates of $P_i$ transport via the $H^+/P_i$ cotransporter. The addition of ouabain to the serosal compartment inhibits the activity of basolateral $Na^+$–$K^+$-ATPase, thus leading to increases of the cytosolic $Na^+$ concentration and reduction of the $Na^+$ gradient across the luminal membrane, which again could reduce the activity of the $Na^+/H^+$ antiporter.

Independent of to what extent and how $Na^+$ may be involved in active $P_i$-transport, it has to be stated that in the present study a proportion (about 30%) of total $J_{net}$ of $P_i$ remained unaffected by any mucosal or serosal manipulations of $Na^+$-dependent $P_i$ transport. From this the existence of further $Na^+$-independent mechanisms for luminal $P_i$-uptake may be surmised.

### Active $P_i$ absorption as affected by dietary calcium and/or phosphorus depletion

Studies in single-stomached species have shown that active $P_i$ absorption can be regulated in response to changes of dietary P intake, and that this effect is mediated by calcitriol (Fox & Care, 1978; Peterlik & Wasserman, 1980; Jungbluth & Binswanger, 1989). Therefore, in the second experiment tissues were taken from goats which had been kept on Ca- and/or P-deficient diets. Significant changes in different variables of Ca and P homeostasis were observed (Table 4). Independent of level of Ca intake P depletion resulted in hypophosphataemia and hypercalcaemia. Increases in plasma Ca levels may have resulted from decreased bone formation and/or increased bone resorption or from respective changes of intestinal Ca absorption and renal Ca secretion (Baylink et al. 1971; Breves & Schröder, 1991). In both Ca-depleted groups increased plasma calcitriol levels occurred which could not be detected when the animals were only P-depleted. The lack of calcitriol stimulation by P-depletion is in contrast to single-stomached species (Baxter & DeLuca, 1976; Fox & Care, 1976; Gray et al. 1983; Breves et al. 1985; Maunder et al. 1986; Shirazi-Beechey et al. 1991). Possibly in P-depleted ruminants the vitamin D-hormone system is regulated at the cytosolic calcitriol receptor level of enterocytes since studies in P-depleted lactating goats have shown increased binding affinities of the receptor (Schröder et al. 1990). This, however, still needs further investigation since in growing goats diet-induced changes of calcitriol receptor properties were not identical to those in lactating goats (unpublished results). Increased plasma PTH concentrations could only be detected in Ca-depleted goats with an adequate P supply, which is similar to single-stomached animals (Chu et al. 1973; Fox, 1992). Possibly the lack of increased PTH levels in the Ca- and P-depleted group was due to hypercalcaemia in those animals (Table 4).

Dietary P depletion resulted in an increased duodenal and jejunal $J_{ms}$ with no significant effect of level of Ca intake (Tables 5 and 6). Since $J_{sm}$ values were not affected, enhanced $J_{ms}$ resulted in increased $J_{net}$, which clearly indicated the stimulation of active $P_i$ transport in response to dietary P depletion. Similar adaptational processes have been described for the duodenum of chicks and rabbits (Quamme, 1985; Danisi et al. 1990) and the jejunum of rats (Caverzasio et al. 1987). In these studies it was demonstrated that increases in $P_i$ uptake were due to increases in $V_{max}$ of the $Na^+/P_i$-cotransport system. Similar findings were obtained by Shirazi-Beechey et al. (1991) in sheep concerning the proposed $H^+/P_i$-cotransport mechanism. In single-stomached species these adaptational processes are controlled by plasma calcitriol (Fuchs & Peterlik, 1980; Matsumoto et al. 1980; Hildmann et al. 1982; Brandis et al. 1987). From the present data it cannot be decided whether the increases in active $P_i$ absorption were due to stimulations of $Na^+$-dependent and/or $Na^+$-independent $P_i$ transport. For both processes it has to be concluded that in goats stimulation of intestinal $P_i$ absorption was not mediated by increased plasma calcitriol or PTH levels since they were not different from control animals. Evidence for calcitriol-independent stimulation of active $P_i$-transport has also been found by Brommage et al. (1990) in rats. Whether other hormones such as insulin (Peterlik et al. 1981), triiodothyronine and thyroxine (Cross et al. 1986), glucocorticoids (Cross et al. 1990) or somatomedins (Gray, 1987) are involved is not clear.

From the present study it may be concluded that in ruminants, as in single-stomached species, $P_i$ can be absorbed actively from the small intestine and that in the mid-jejunum

of sheep a substantial portion of the active transport consists of a $Na^+$-dependent $P_i$-transport mechanism. Further details on potential differences between intestinal segments and ruminant species have not yet been clarified. At present it is not clear by which mechanism the remaining 30% of $J_{net}$ of $P_i$ was mediated, and it still has to be ascertained whether the increased transport rates in response to dietary P depletion were due to changes of the $Na^+$-dependent and/or $Na^+$-independent mechanisms and how these changes were induced at the cellular level.

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