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Ex vivo intestinal studies on calcium and phosphate transport in growing goats fed a reduced nitrogen diet

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Abstract

In ruminant feeding, the reduction of dietary protein is an effective approach for decreasing the excretion of N. In non-ruminant species, the intestinal absorption of Ca was affected when dietary protein was reduced. Therefore, it was the aim of the present study to characterise the intestinal absorption of Ca and inorganic phosphate (P_i) in goats fed different N and Ca diets. Intestinal flux rates of Ca and P_i were determined in goats fed a reduced N and Ca diet by Ussing chamber experiments. For a more mechanistic approach, the uptake of Ca and P_i in intestinal brush-border membrane vesicles (BBMV), the expression levels of the epithelial Ca channel transient receptor potential vanilloid channel type 6 (TRPV6), the sodium-dependent P_i transporter (NaPi) IIb and the vitamin D receptor (VDR) were measured. In goats fed a reduced N and Ca diet, the intestinal flux rates of Ca and P_i were elevated. However, the reduced N and Ca diet had no effect on the uptake of Ca and P_i in intestinal BBMV, while the expression of TRPV6 and NaPi IIb protein in the corresponding intestinal segments was even decreased. The mRNA expression of NaPi IIb and VDR was not affected. Therefore, a post-transcriptional regulation of TRPV6 and NaPi IIb protein was suggested in goats fed a reduced N and Ca diet. From these data, it can be concluded that the intestinal absorption of Ca and P_i in growing goats was affected by changes in dietary N and Ca intake like those in single-stomached animals but differently modulated.

Key words: Flux rates of calcium and phosphate: Goats: Sodium-dependent phosphate transporter IIb: Transient receptor potential vanilloid channel type 6: Ussing chambers

In ruminants, feeding low-protein diets is desirable to reduce N excretion of the animals and thereby decrease the output of N into the environment. Since excretion of N is directly correlated with N intake, a decrease of urinary N excretion was observed in goats when dietary N intake was reduced[1,2]. Ruminants like goats are able to recycle N efficiently, in contrast to single-stomached animals, by rumino-hepatic circulation. During dietary N reduction, the renal tubular absorption of urea and the ruminal urea transport capacity were increased[3,4]. These adaptive responses ensure an efficient rate of protein synthesis by rumen micro-organisms, whereby microbial protein serves as an amino acid source for the host, especially when dietary N is deficient. Therefore, ruminants are unique in their capability to cope with reduced N intake. In contrast, single-stomached animals and humans are not able to utilise endogenous N sources when consuming a low-protein diet.

In single-stomached animals and humans, metabolic responses to a reduction in dietary protein supply resulted in stunted growth, decreased plasma urea concentrations, diminished urinary excretion of urea and reduced serum insulin-like growth factor-1 (IGF-1) concentrations[5–7]. Furthermore, evidence demonstrated that protein metabolism in these species was closely related to other homeostatic systems such as Ca homeostasis. In rats, a low-protein diet was associated with hypocalciuria, decrease of plasma calcitriol (1,25-dihydroxyvitamin D_3) concentration and reduced intestinal absorption of Ca[8,9]. In humans consuming a low-protein diet, intestinal Ca absorption rates were reduced and plasma parathyroid hormone concentrations were increased[10].

Concentrations of 11–12% crude protein (CP) in the diet were suggested to be adequate to meet the requirements for moderate growth performance of young goats[11]. Although goats, like all other ruminants, are able to maintain their

Abbreviations: BBMV, brush-border membrane vesicles; BW, body weight; CP, crude protein; FE, fractional excretion; G_T, tissue conductance; IGF-1, insulin-like growth factor-1; I_{SC}, short-circuit current; J_{net}, net flux rates; J_{ms}, unidirectional flux rates from mucosal to serosal; J_{sm}, unidirectional flux rates from serosal to mucosal; NaPi IIb, sodium-dependent phosphate transporter; P_i, inorganic phosphate; TRPV6, transient receptor potential vanilloid channel type 6; VDR, vitamin D receptor.

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N balance of the whole body when fed low-protein diets, the question arises if other processes could be affected like those observed in single-stomached species. This would limit the possibility to reduce N intake for decreasing environmental pollution due to potential detrimental effects on the metabolic homeostasis and health in ruminants. Interestingly, in two previous studies[12,13], we demonstrated that reducing the dietary N supply under normocalcaemic and hypocalcaemic conditions had a significant effect on electrolyte metabolism in growing goats, mainly reflected in a decrease of plasma calcitriol concentrations. This reduction of plasma calcitriol is suggested to have an inhibitory effect on the intestinal absorption of Ca and inorganic phosphate (P_i) in ruminants, because it is hypothesised that these processes are calcitriol-dependent like in single-stomached animals[14–16]. Consumption of a low-Ca diet, however, led to an increase of plasma calcitriol concentrations which stimulated the intestinal absorption of Ca in single-stomached animals and humans[17,18]. Therefore, the question arises if the reduction in plasma calcitriol concentrations resulting from a reduced N diet can be counteracted by the stimulation of calcitriol production by a simultaneous decrease in dietary N and Ca, and if this influences the intestinal electrolyte absorption.

To elucidate this network of N metabolism and electrolyte homeostasis, growing goats were challenged with two antidromic dietary stimuli, a simultaneous reduction in N and Ca intake, to characterise the transport of Ca and P_i in the small intestines. For this purpose, unidirectional flux rates of Ca and P_i across the intestinal epithelia from goats fed diets with different N and Ca content were determined in Ussing chambers. The uptake of Ca and P_i into isolated brush-border membrane vesicles (BBMV) and the expression of intestinal transient receptor potential vanilloid channel type 6 (TRPV6), Na^+-dependent P_i transporter (SLC34A2, NaPi IIb) and vitamin D receptor (VDR) were examined for a more detailed mechanistic approach.

Materials and methods

The animal feeding and handling regimes were approved by the Animal Welfare Commissioner of the University of Veterinary Medicine Hannover in accordance with the German Animal Welfare Law.

Animals and feeding regimes

After weaning (6 weeks postnatal), twenty male White Saanen goats, purchased from a commercial breeder farm, were separated from their mothers and housed in pens bedded with sawdust. For 2 weeks, all animals were fed a mixture composed of all three concentrate diets and straw in a 4:1 ratio with a CP content of 12% to allow the goats to acclimatise to pelleted diets. At the age of about 2 months with initial weights of 16·5 (SE 0·5) kg, the goats were allocated to a diet containing either 19% CP and 0·7% Ca (19% CP; *n* 7), 10% CP and 0·6% Ca (10% CP; *n* 7) or 7% CP and 0·5% Ca (7% CP; *n* 6), for 7–9 weeks. To maintain the Ca:P_i ratio in the diets, P_i was adjusted. The contents of each diet were assessed on an as-fed basis. The animals were housed in groups of six to seven on wooden shavings with water available *ad libitum*. The fed amount of concentrate per animal and day was 70 g/kg metabolic body weight ($BW^{0·75}$). Additionally, 33% of the concentrate weight was given as chopped wheat straw and feeding was performed three times daily. Food was placed in broad feeding troughs to allow undisturbed food intake for each animal in the group. The total amounts of all feeds offered and refused were monitored daily to estimate the mean daily intake of nutrients and minerals over the entire experimental period (Table 2).

Diets

Dietary ingredients have been described in detail elsewhere[3,12]. The feed contents of DM, crude ash, crude fibre, crude fat and CP were determined by standard procedure in accordance with the methods of the Association of German Agricultural Investigation and Research Centre[19]. The components and composition of wheat straw and concentrates are presented in Table 1. Sipernat 22S, a fine particle silica, which cannot be metabolised, was used to adjust the weight of reduced N diets.

Blood and urine samples

Plasma samples were taken 24 h before slaughter. Blood samples (9 ml each) were taken by venepuncture from the vena jugularis with EDTA or lithium heparinate covered syringes (Sarstedt, Nümbrecht, Germany). Blood plasma was separated by centrifugation (2000 ***g*** at room temperature for 15 min) and stored at − 20°C. Urine samples were collected during slaughter by aspiration from the bladder. For one goat in the 19% CP group, no urine was present in the bladder.

Intestinal tissue samples

At the end of the study, the animals (3–4 months of age) were slaughtered by exsanguination after captive bolt stunning. Within 3–5 min after slaughter, segments of about 60 cm in length from the proximal and mid-jejunum were removed from the abdominal cavity beginning 1 m distal from the pylorus. Intestinal segments were rinsed with ice-cold saline (0·9% w/v) and kept in a glucose-containing Krebs-Henseleit buffer solution, continuously aerated with carbogen (95% O_2–5% CO_2) until the tissue preparations were mounted in Ussing chambers[20,21]. For RNA isolation, nuclear extracts and preparation of plasma membrane-enriched fractions, samples were rinsed with ice-cold saline (0·9% w/v). The mucosa of the proximal and mid-jejunum was stripped off, frozen in liquid N_2 and stored at −80°C immediately.

Intestinal flux rate measurements of calcium and inorganic phosphate

Determination of Ca, P_i and mannitol flux rates across the intestinal epithelia was performed in Ussing chambers with an exposed surface of 1·13 cm^2 under short-circuit current

Table 1. Components and composition of wheat straw and pelleted concentrate diets*

	Wheat straw	19 % CP	10 % CP	7 % CP
Components, g/kg (as-fed basis)				
Beet pulp	–	425	425	455
Tapioca	–	407	407	452
Soyabean meal	–	108	108	33
Soyabean oil	–	10	10	10
Mineral–vitamin mix†	–	10	10	10
$MgHPO_4 \cdot 3H_2O$	–	4	4	6
Urea	–	30	–	–
Sipernat 22S‡	–	6	36	34
Composition				
DM (g/kg)	907	966	962	961
Nutrient (g/kg DM)				
Crude ash	56·2	58·0	87·3	89·5
CP	37·5	200	101	69·7
Crude fat	6·6	22·8	19·8	17·7
Urea	BDL	32·1	BDL	BDL
Ca	2·8	7·1	6·1	4·6
P	0·8	2·8	2·5	2·1
Vitamin D_3	BDL	BDL	BDL	BDL
DCAD (mEq/kg DM)	–	210	210	200
ME (MJ/kg DM)	6·4	13·0	12·2	12·1

CP, crude protein; BDL, below detection level; DCAD, dietary cation anion difference; ME, metabolisable energy.
* Composition expressed as fed.
† Mineral–vitamin mix per kg: 180 g Ca; 60 g P; 100 g Na^+; 30 g Mg; 500 000 IU (525 μmol/l) vitamin A; 80 000 IU (4992 nmol/l) vitamin D_3; 300 mg (697 μmol/l) vitamin E; 4200 mg Zn; 900 mg Mn; 16 mg Co; 20 mg iodine; 44 mg Se.
‡ Sipernat type 22S (Evonik Industries AG, Essen, Germany) is a fine particle silica with high oil absorption capacity. It is widely used as flow regulator, anti-caking and dusting agent especially in the food and feed industry.

conditions as described by Schröder *et al.*[20,21]. The tissue conductance and the short-circuit current were determined by a computer-controlled voltage clamp device (Mussler Scientific Instruments, Aachen, Germany). Tissues were incubated on both sides with a 10 ml buffer solution (adjusted under carbogen saturation to pH 7·4 at 38°C with HCl) containing (mM): 113·6 NaCl, 5·4 KCl, 1·2 $MgCl_2.6H_2O$, 21·0 $NaHCO_3$, 1·2 $CaCl_2.2H_2O$, 1·2 $Na_2HPO_4.2H_2O$ and 1·2 mannitol, respectively. The serosal buffer additionally contained 10·0 mM-glucose and 0·01 mM-indomethacin. During the experiments, the buffers were continuously aerated with carbogen at 38°C. After the tissue had been equilibrated for 30 min, ^{45}Ca, ^{32}P and (^3H)-mannitol (each about 185 kBq/chamber) were used as radioisotopic tracers (Hartmann, Brunswick, Germany). Samples were taken at intervals of 10 min and replaced with equal volumes of the buffer solution. Radioactivity of the samples was measured by a liquid scintillation counter (Tri-Carb 2500 TR Packard Instruments Company, Downers Grove, IL, USA). The (^3H)-mannitol was used as a paracellular marker[22]. Unidirectional flux rates from mucosal to serosal (J_{ms}) and serosal to mucosal (J_{sm}) of Ca, P_i and mannitol were calculated from the rate of tracer appearance on the unlabelled side using standard equations[23]. Net flux rates (J_{net}) were determined as differences between J_{ms} and J_{sm} of paired tissues.

Biochemical determinations

Haematocrit was determined in whole blood samples. An aliquot of each blood sample was placed in a microhaematocrit tube and spun to constant packed cell volume at 14 926 ***g*** for 5 min at room temperature. Plasma and urine urea concentrations were measured using a commercial kit (R-Biopharm, Darmstadt, Germany). The inter- and intra-assay CV for urea were 2·7 and 1 %, respectively. Plasma and urine creatinine were analysed by a standard diagnostic method in the Clinic for Diseases of Cattle at the University of Veterinary Medicine in Hannover. Total plasma protein concentration was measured with a commercial Coomassie blue protein assay (Bio-Rad, Munich, Germany) using bovine plasma gamma globulin as standard protein. Inter- and intra-assay CV of protein were 1·2 and 1·0 %, respectively. Plasma albumin concentrations were detected by a standard dye binding technique using bromocresol green (Hengler Analytik, Steinbach, Germany). Inter- and intra-assay CV of albumin were 7·7 and 2·3 %, respectively. Ionised Ca was measured in whole blood samples using an ion-sensitive electrode (Chiron Diagnostics GmbH, Wiesentheid, Germany). Inter- and intra-assay CV of ionised Ca were 2 and 1 %, respectively. Concentrations of total Ca and inorganic P_i were measured colorimetrically in plasma and urine by standard spectrometric techniques[24,25]. Inter- and intra-assay CV of total Ca were 4·4 and 2·4 % and for P_i they were 3·7 and 1·9 %, respectively. Na^+ concentrations in plasma and urine were detected using a Na^+-selective electrode (ABX Pentra 400; Horiba ABX, Montpellier, France). Inter- and intra-assay CV of Na^+ were 0·4 and 0·2 %, respectively. The concentration of calcidiol was determined by a competitive EIA (Immundiagnostik AG, Bensheim, Germany). The declared inter- and intra-assay CV of the kit were $<13·2$ and $<10·7$ %, respectively. Calcitriol concentrations were measured by a commercial radioreceptor assay (Immundiagnostik AG). The intra-assay CV were <15 and <10 % for samples with calcitriol concentrations of 10 and 60 pg/ml, respectively. The inter-assay CV were <20

and <15 % for these two concentrations. The detection limit of this assay was 2 pg/ml. The calcitriol assay systems had already been used in other studies to determine the concentration of this hormone in goat plasma[26–28]. For total plasma IGF-1 determination, an ACTIVE IGF-1 coated tube IRMA Kit (DSL-5600; Diagnostic Systems Laboratories, Inc., Webster, TX, USA) was used. The IGF-1 was separated from its binding proteins by an acid–ethanol extraction procedure, and IGF-1 concentrations were determined with a two-site immunoradiometric assay. The intra-assay CV was 1·5–3·5 % and the inter-assay CV was 1·5–8·5 %. The amount of growth hormone was measured by an ELISA as previously described[13,29,30]. Intra- and inter-assay CV were 9·8 and 12·6 %, respectively. The lowest detection limit was 1·0 ng/ml and the ED_{50} in this assay system was 7·6 ng/ml.

Calculation of fractional excretion

The fractional excretion (FE) of urea was determined at the end of the experimental period using the following formula:

$$\text{FE urea} = \frac{\text{Creatinine}_{\text{plasma}}\,(\text{mM}) \times \text{Urea}_{\text{urine}}\,(\text{mM})}{\text{Creatinine}_{\text{urine}}\,(\text{mM}) \times \text{Urea}_{\text{plasma}}\,(\text{mM})} \times 100\,\%.$$

The FE of total Ca, P_i and Na^+ was calculated by applying the same equation, but with the respective values for total Ca, P_i and Na^+. The reliability of creatinine for measuring the renal function during protein reduction was suitably shown by Valtonen *et al.*[31].

Isolation of intestinal brush-border membrane vesicles, calcium and inorganic phosphate uptake studies

The preparation of small-intestinal BBMV, Ca and P_i uptake studies have already been described by Schröder *et al.*[27] and Muscher *et al.*[26]. Briefly, BBMV from proximal and mid-jejunum were prepared according to a Mg^{2+} precipitation method, with two precipitation steps.

The uptake of Ca in BBMV of proximal jejunum was performed using a method described by Kaune *et al.*[32] with slight modifications. For the uptake of ^{45}Ca/Ca, BBMV of proximal jejunum were incubated at 21°C, with the uptake medium containing 75 mM-mannitol, 75 mM-KCl, 7·5 mM-HEPES–Tris, 0·5 mM-ethylene glycol tetraacetic acid, non-labelled Ca and 37 kBq/incubation vessel ^{45}Ca (Hartmann). The desired free Ca concentrations were obtained by adding appropriate amounts of Ca to 0·5 mM-ethylene glycol tetraacetic acid. The amounts of Ca were calculated by Winmax32 version 2.50 (Chris Patton, Stanford University, USA; http://www.stanford.edu/~cpatton/maxc.html). The concentration-dependent Ca uptakes were performed over a range of 0·045–4·0 mM-Ca.

For the uptake of $^{32}P/P_i$, BBMV of mid-jejunum were incubated at 21°C with the uptake medium containing non-labelled P_i and α ^{32}P (Hartmann; 37 kBq/incubation vessel). Concentration-dependent P_i uptakes were performed over a range of 0·01–1·0 mM-P_i. Extravesicular incubation buffer contained 100 mM-mannitol, 10 mM-HEPES–Tris, pH 7·4, and 100 mM-NaCl. The Na^+ dependency of P_i transport was established by incubating BBMV of mid-jejunum in solutions in which KCl replaced NaCl equimolarly. The proximal BBMV were washed with a stop solution containing 150 mM-KCl, 50 mM-mannitol, 10 mM-HEPES–Tris and 1 mM-ethylene glycol tetraacetic acid, pH 7·4, while the BBMV from mid-jejunum were washed with 150 mM-KCl, 1 mM-KH_2PO_4 and 10 mM-HEPES–Tris, pH 7·4, both on 0·65 μm cellulose nitrate filters. The activity of each filter was counted using a Packard Tri-Carb 2500TR scintillation counter. Kinetic parameters V_{max} (nmol Ca/(mg protein × 30 s) or nmol P_i/(mg protein × 10 s)) and K_m (mM) were calculated from the Michaelis–Menten kinetic of Ca or P_i uptake into the intestinal BBMV.

Intestinal expression of sodium-dependent phosphate transporter IIb and vitamin D receptor mRNA in goats fed a 19 % crude protein v. 7 % crude protein diet

Semi-quantitative detection of specific amounts of NaPi IIb and VDR mRNA in caprine proximal and mid-jejunum was performed via Northern blot analysis as described in detail elsewhere[26,33]. Briefly, isolated 6 μg poly(A)$^+$RNA/lane from proximal or mid-jejunum was fractionated in 1·0 % formamide/agarose gels and transferred by capillary blotting onto nitrocellulose membranes. Radioactive ^{32}P-labelled NaPi IIb, VDR- and ß-actin-specific probes were hybridised to fixed mRNA. Hybridisation took place for 16 h at 42°C (40 % formamide). The membranes were analysed with a phosphorous imager system (Bio-Rad) after exposure to a phosphorous imager screen for 2–6 h. The relative amounts of specific mRNA were quantified by reference to β-actin as an internal standard using the quantification software Quantity One (Bio-Rad).

Intestinal expression of transient receptor potential vanilloid channel type 6, sodium-dependent phosphate transporter IIb, Na^+K^+ATPase and vitamin D receptor protein in goats fed a 19 % crude protein v. 7 % crude protein diet

Mucosa samples from proximal and mid-jejunum were homogenised and crude membranes prepared by performing differential centrifugation[34]. From mid-jejunum, BBM were isolated using the Mg^{2+} precipitation method previously described. The isolation of nuclear extracts from caprine proximal and mid-jejunum was carried out by a method described by Schröder *et al.*[35] and Muscher *et al.*[26]. The protein concentrations of all preparations were determined by the Bradford method (Bio-Rad). Immunoblot assays detecting TRPV6, NaPi IIb and VDR proteins in caprine intestinal tissues were performed as previously described[26,34]. For the abundance of TRPV6, NaPi IIb and Na^+K^+ATPase, 15–50 μg of enriched plasma membranes or BBM were separated by 8·5 % SDS–PAGE and transferred to nitrocellulose membranes (GE Healthcare, Freiburg, Germany) using a tank blotting system (Bio-Rad). For the detection of VDR, 15 μg of nuclear extracts were separated in 10 % SDS–polyacrylamide gels in the same manner. Nitrocellulose membranes were blocked

Table 2. Effects of a reduced nitrogen and calcium diet on the performance of growing goats as estimated from pooled group mean values over the entire experimental period

(Mean values)

Item	19 % CP	10 % CP	7 % CP
n	7	7	6
DM intake (g/d)*	701	751	558
Concentrate intake (g/d)†	598	599	431
N intake (g/d)*†	18·7†	10·2	5·5
Ca intake (g/d)†	4·5	4·0	2·3
P intake (g/d)	1·7	1·6	1·0
Initial body weight (kg)	16·5	16·7	16·2
Final body weight (kg)	21·8	23·4	19·8
Body weight gain (kg/d)*	0·101	0·137	0·059

CP, crude protein.
* Results have previously been published in Muscher *et al.*[3].
† Results in the 19 % CP and 7 % CP columns were previously published in Muscher *et al.*[12].

overnight at 4°C in PBS containing fat-free milk powder. Immunodetection of electrotransferred proteins was performed according to standard procedures. The following primary antibodies were used: anti-TRPV6 (H-90; Santa Cruz Biotechnology, Heidelberg, Germany), anti-NaPi IIb (gift from Professor Dr J. Biber), anti-VDR (Enzo Life Sciences GmbH, Lörrach, Germany), anti-villin (Biotrend Chemikalien GmbH, Cologne, Germany) and anti-Na^+K^+ATPase (Enzo Life Sciences GmbH). Immunoreactive proteins were visualised using the enhanced chemiluminescence system (Perbio Science GmbH, Bonn, Germany) according to the manufacturer's protocol. Villin was used as an internal standard to semiquantify relative protein expression amounts. Bands were analysed semiquantitatively using the Quantity One software (Bio-Rad).

Statistical analysis

All data are expressed as means with their pooled standard errors, with number of animals. Data were analysed by one-way ANOVA with Tukey's post test except Northern and Western Blot analyses which were analysed by unpaired Student's *t* test. Kinetic parameters of Ca or P_i uptake into isolated BBMV were calculated using non-linear regression analysis. Potential relationships between the measured parameters were calculated by linear regression analysis. All statistical analyses were performed using GraphPad Prism version 4·0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). In all cases, $P<0·05$ was set to be significantly different and $P<0·1$ was used to define trends.

Results

Intake, body weight and daily weight gain

Daily DM, concentrate, N, Ca and P intake on a per-animal basis were estimated from group mean values. Mean daily DM, concentrate, N, Ca and P intake, BW gain, and BW over the entire experimental period are summarised in Table 2. Some of these results have already been published by Muscher *et al.*[3,12].

Blood parameters as affected by the reduction of dietary nitrogen and calcium in growing goats

The haematocrit, total plasma protein and albumin content in the growing goats fed a reduced N and Ca diet remained unchanged throughout the complete experimental period,

Table 3. Effects of a reduced nitrogen and calcium diet on blood parameters of growing goats*

(Mean values and pooled standard errors)

Item	19 % CP†	10 % CP	7 % CP†	SEM	*P*
n	7	7	6		
Total blood					
Haematocrit (%)	26·6	27·3	27·6	2·09	0·86
Ionised Ca (mM)	1·40[a]	1·29[b]	1·26[b]	0·047	0·006
pH	7·42	7·42	7·41	0·013	0·42
Plasma					
Total protein (mg/ml)	69·8	70·3	68·6	3·26	0·81
Albumin (g/l)	28·9	31·0	28·4	2·66	0·45
Total Ca (mM)	2·66[a]	2·50[a]	2·32[b]	0·12	0·013
P_i (mM)	1·71	1·79	1·89	0·30	0·78
Na^+ (mM)	141·7	140·9	143·2	1·32	0·13
Calcidiol (mM)	14·66	16·94	18·22	4·92	0·68
Calcitriol (pg/ml)	46·80	39·89	31·63	7·07	0·06
Growth hormone (ng/ml)	7·3	6·7	12·8	5·61	0·38
IGF-1 (ng/ml)	181[a]	175[a]	46·2[b]	51	0·009
Fractional excretion of (%)					
n	6	7	6		
Urea	93·4[a]	43·3[a,c]	7·9[b,c]	27·8	0·007
Ca	3·12[a]	1·12[b]	1·26[b]	0·85	0·02
P_i	1·16	0·97	0·77	0·20	0·10
Na^+	0·39	0·77	0·54	0·51	0·65

CP, crude protein; IGF-1, insulin-like growth factor-1; P_i, inorganic phosphate.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Fractional excretion of urea, Ca, P_i and Na^+ of goats fed different N and Ca supply.
† Values in the 19 % CP and 7 % CP columns for ionised Ca, total CA, P_i, calcidiol and calcidiol have previously been published in Muscher *et al.*[12].

Table 4. Transepithelial conductance (G_T, in mS/cm^2), short-circuit current (I_{SC}, in μEq/(cm^2 × h)), mucosal-to-serosal (J_{ms}) in nmol/(cm^2 × h), serosal-to-mucosal (J_{sm}) in nmol/(cm^2 × h) and net flux rates ($J_{net} = J_{ms} - J_{sm}$) flux rates of calcium, inorganic phosphate (P_i) and mannitol (man) in goat proximal jejunum and mid-jejunum as affected by dietary nitrogen and calcium supply

(Mean values and pooled standard errors)

Item	19 % CP	10 % CP	7 % CP	SEM	*P*
n	7	7	6		
Proximal jejunum					
G_T	11·8	13·3	14·8	1·75	0·14
I_{SC}	0·42^a	0·48^a	1·29^b	0·22	<0·001
J_{ms} Ca	35·0^a	39·4^a	76·1^b	9·35	<0·001
J_{sm} Ca	31·0	27·3	32·7	5·95	0·54
J_{net} Ca	4·08^a	12·0^a	43·4^b	6·06	<0·001
Mid-jejunum					
G_T	10·7	11·8	14·5	2·10	0·11
I_{SC}	0·63^a	0·64^a	1·25^b	0·17	<0·001
J_{ms} Ca	35·4	35·3	34·6	5·12	0·98
J_{sm} Ca	38·8	37·9	26·3	6·38	0·06
J_{net} Ca	−3·40^a	−2·58^a	8·26^b	4·22	0·007
J_{ms} Pi	132	146	213	40·4	0·06
J_{sm} Pi	37·8^a	34·8^{a,b}	28·5^b	4·23	0·049
J_{net}Pi	94·5^a	111^{a,b}	184^b	41·7	0·045
J_{ms} man	48·2^{a,b}	44·4^a	58·2^b	5·77	0·029
J_{sm} man	36·8^a	32·6^{a,b}	22·6^b	5·43	0·017
J_{net} man	11·4^a	11·8^a	35·7^b	9·28	0·009

CP, crude protein.
^{a,b} Mean values within a row with unlike superscript letters were significantly different (P<0·05).

indicating that there was neither haemoconcentration nor haemodilution with the various diets (Table 3). Both, total and ionised plasma Ca concentrations were significantly reduced, while blood pH was not influenced in the goats fed a reduced N diet with varied Ca content[12] (Table 3). The concentrations of plasma P_i and Na^+ were not affected by changes in this feeding regime in the young goats (Table 3). Plasma calcidiol concentrations remained unchanged, while calcitriol concentrations tended to be reduced (P=0·06) when dietary N was decreased (Table 3). The decline in plasma IGF-1 concentrations was positively related to plasma urea levels (r^2 0·278, P=0·021) while concentrations of growth hormone were not affected (Table 3). Some of these blood parameters have already been published in the work by Muscher *et al.*[12].

Fractional excretion of urea, calcium, inorganic phosphate and sodium as affected by the reduction of dietary nitrogen and calcium in growing goats

The reduction of dietary N and Ca resulted in decreases of FE of urea and Ca. The FE of P_i tended to be reduced (P=0·10) while the FE of Na^+ remained unaffected (Table 3).

Electrophysiological properties as affected by the reduction of dietary nitrogen and calcium in growing goats

For both epithelia, tissue conductance was not affected by changes in the diet, while basal short-circuit current of the 7 % CP group in both the intestinal segments increased in comparison with the two other feeding groups (Table 4).

Flux rates of calcium and inorganic phosphate across intestinal mucosa as affected by the reduction of dietary nitrogen and calcium in growing goats

In growing goats fed a reduced N and Ca diet, the J_{ms} flux rates of Ca in the proximal jejunum were elevated in the animals of the 7 % CP group without having any effect on the J_{sm} flux rates, resulting in increased J_{net} in these animals in comparison with the 19 % CP and 10 % CP groups (Table 4). In mid-jejunum, the J_{ms} flux rates of Ca were not influenced by the dietary treatment while J_{sm} flux rates of Ca were only tendentially affected, which still resulted in an increase of J_{net} flux rates for the animals in the 7 % CP group in comparison with both other groups (Table 4). The Na^+-dependent P_i transport was measured in mid-jejunum as the main absorption site for P_i in goats[20,33]. A transfer of P_i across the intestinal epithelium from the mucosal to the serosal side as well as from the serosal to the mucosal side could be detected in all animals (Table 4). The reduction of dietary N and Ca supply increased the J_{ms} flux rates of P_i while the J_{sm} flux rates were decreased, which resulted in significantly higher J_{net} flux rates of P_i in the goats fed a 7 % CP diet (Table 4). The changes of the dietary N and Ca content had an impact on

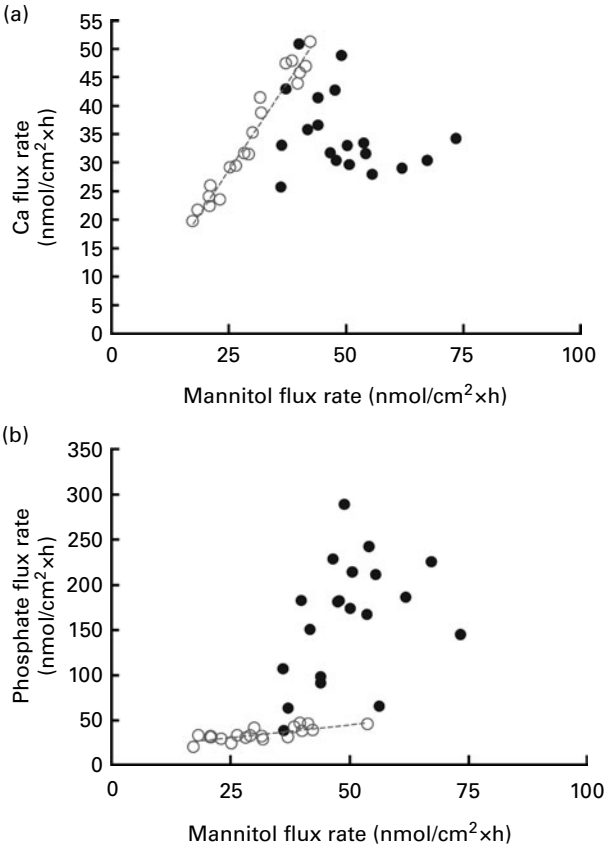

Fig. 1. Linear regression of unidirectional mucosal to serosal (J_{ms}; ●) or serosal to mucosal (J_{sm}; ○, -----) flux rates of (a) calcium (J_{sm} Ca = (1·24 ± 0·06) J_{sm} mannitol − (2·33 ± 1·96), r^2 0·96, P<0·001) or (b) inorganic phosphate (P_i; J_{sm} P_i = (0·56 ± 0·11) J_{sm} mannitol+ (16·45 ± 3·59), r^2 0·59, P<0·001) with the corresponding mannitol flux rates in mid-jejunum of goats fed different nitrogen and Ca diets. Calculations were only provided when significance was obtained by linear regression.

the mannitol flux rates in mid-jejunum, too. The J_{ms} flux rates of mannitol were elevated in the 7% CP group in comparison with the other two diets while the J_{sm} flux rates of mannitol were reduced, causing an increase of J_{net} flux rates of mannitol in the 7% CP group (Table 4). High significant correlations between J_{sm} mannitol and J_{sm} Ca flux rates ($P<0{\cdot}001$; Fig. 1(a)) and between J_{sm} mannitol and J_{sm} P_i flux rates ($P<0{\cdot}001$), respectively, could be revealed by linear regression (Fig. 1(b)).

Intestinal uptake of calcium and inorganic phosphate in isolated brush-border membrane vesicles as affected by the reduction of dietary nitrogen and calcium in growing goats

The functional integrity of the isolated BBMV from proximal and mid-jejunum was tested by performing a respective successful characteristic Na^+-dependent overshoot phenomenon of glucose uptake (data not shown). The reduction of dietary N and Ca had no effect on the kinetic parameters like transport capacity (V_{max}) and transporter affinity (K_m) of Ca and P_i uptake in the isolated BBMV of proximal and mid-jejunum (Table 5).

Intestinal expression of sodium-dependent phosphate transporter IIb and vitamin D receptor mRNA as affected by the reduction of dietary nitrogen and calcium in growing goats

A reduction of dietary N and Ca caused a tendential increase of NaPi IIb-specific mRNA in mid-jejunum, while neither the expression patterns nor the expression amounts of VDR-specific mRNA in proximal and mid-jejunum were affected in the growing goats (Table 5).

Intestinal expression of transient receptor potential vanilloid channel type 6, sodium-dependent phosphate transporter IIb, vitamin D receptor and Na^+K^+ATPase protein as affected by the reduction of dietary nitrogen and calcium in growing goats

The protein expression of the Ca channel, TRPV6, was significantly reduced in the crude membranes of proximal jejunum in goats in the 7% CP group in comparison with the 19% CP group (Table 5). The reduction of dietary N and Ca content had no impact on the protein amount of the nuclear located VDR either in proximal or in mid-jejunum in the growing goats (Table 5). Changes in the Na^+K^+ATPase protein expression could not be detected in the crude membranes of proximal and mid-jejunum (Table 5). In mid-jejunum, the protein expression of the Na^+-dependent P_i transporter, NaPi IIb, was significantly reduced in the 7% CP group in comparison with the animals fed 19% CP (Table 5). Additionally, the NaPi IIb protein expression was positively correlated with the plasma calcitriol concentrations of these animals (r^2 0·353, $P=0{\cdot}032$).

Discussion

The aim of the present study was to investigate whether the intestinal absorption of Ca and P_i is modulated by a concomitant reduction of dietary N and Ca supply in goats. With the present study, it could be shown for the first time that a reduction of N and Ca intake has an impact on Ca and P_i absorption in caprine intestinal epithelia. However, limitations of the present study were that the effects of a dietary N or Ca reduction were not examined separately. No individual feed intakes of the goats were monitored, but the animal was still acceptable as the experimental unit for all other data of this

Table 5. Relative amounts of sodium-dependent phosphate transporter (NaPi) IIb and vitamin D receptor (VDR) mRNA expression as well as relative amounts of NaPi IIb, transient receptor potential vanilloid channel type 6 (TRPV6), VDR and Na^+K^+ATPase protein expression in proximal and mid-jejunum in goats fed different nitrogen and calcium diets*

(Mean values with their standard errors)

Item	19% CP	10% CP	7% CP	SEM	*P*
n	7	7	6		
Proximal jejunum					
V_{max} (nmol Ca/(mg protein × 30 s))	4·29	6·88	8·14	2·56	0·20
K_m (mmol Ca/l)	3·44	6·89	8·33	2·90	0·14
VDR mRNA:ß-actin mRNA ratio	0·31	ND	0·32	0·03	0·98
TRPV6 protein:villin protein ratio	0·77	ND	0·40	0·15	0·03
VDR protein:villin protein ratio	1·29	ND	1·45	0·33	0·64
Na^+K^+ATPase protein:villin protein ratio	0·87	ND	1·01	0·13	0·30
Mid-jejunum					
V_{max} (nmol P_i/(mg protein × 10 s))	0·51	0·56	0·46	0·10	0·52
K_m (mmol P_i/l)	0·04	0·04	0·03	0·01	0·77
NaPi IIb mRNA:ß-actin mRNA ratio	1·11	ND	1·42	0·16	0·08
VDR mRNA:ß-actin mRNA ratio	0·18	ND	0·22	0·03	0·25
NaPi IIb protein:villin protein ratio	2·10	ND	0·84	0·46	0·02
VDR protein:villin protein ratio	2·41	ND	2·19	0·36	0·56
Na^+K^+ATPase protein:villin protein ratio	4·26	ND	4·77	1·25	0·69

CP, crude protein; V_{max}, maximal transport rate; K_m, half-maximal saturation value; ND, not determined; P_i, inorganic phosphate.
* Kinetic parameters, V_{max} and K_m, of Ca and Na^+-dependent P_i uptake in caprine small-intestinal brush-border membrane vesicles of goats fed different N and Ca diets.

study because final BW of each animal, which was recorded individually, was not affected while plasma urea concentrations were concomitantly diminished like the percentage of N in the diets. For this reason, we decided to calculate with individual animals as animals having received a group-specific treatment (19% CP, 10% CP and 7% CP).

To characterise the metabolic status of the goats fed a reduced N and Ca diet, concentrations of IGF-1, growth hormone and calcitriol were determined. All three parameters were consistent with the concentrations from goats fed a reduced N diet under normocalcaemic conditions[13].

In proximal jejunum, the major intestinal site for Ca absorption in small ruminants[34,36], the J_{ms} flux rates of Ca were significantly increased, while the J_{sm} flux rates of Ca remained constant. This resulted in significant increases in J_{net} flux rates, indicating a stimulatory effect on active Ca transport in response to reductions in N and Ca intake. In order to differentiate between the para- and transcellular component of unidirectional Ca flux rates, the paracellular marker mannitol was used as introduced by Auchere *et al.*[22]. For technical reasons, mannitol flux rates could only be measured in mid-jejunum. At this site, Ca flux rates from the serosal to the mucosal side correlated significantly with the respective mannitol flux rates, whereas no correlation could be detected for J_{ms} (Fig. 1(a)). From these correlations it can be concluded that these Ca flux rates were mainly mediated by active, transcellular transport mechanisms. Suggesting that the transcellular pathway was the major route for J_{ms} Ca flux rates, the dietary reduction of N and Ca would have had a stimulatory effect on the active absorption of Ca in proximal jejunum of growing goats.

However, the mechanisms by which the modulation of intestinal electrolyte transport in response to reduced N and Ca intake are mediated are not yet understood. Interestingly, a reduced N and Ca diet led to low plasma Ca concentrations, which were not observed in goats or rats fed a low-Ca diet only[36,37]. Physiologically, a low-Ca diet led to elevated plasma calcitriol concentrations in single-stomached animals and ruminants[36,37]. An increase in plasma calcitriol is responsible for the stimulation of intestinal Ca absorption in single-stomached animals[37]. In sheep, the stimulation of jejunal Ca absorption was only detected in response to supraphysiological dosage of exogenous calcitriol[34]. When only dietary Ca intake was reduced, no effects on intestinal Ca transport were detected in goats and sheep[34,36]. Therefore, a stimulation of endogenous calcitriol production by the diet has modulatory effects on the intestinal absorption of Ca in single-stomached animals. However, this effect could not be seen in small ruminants. In contrast, the findings of the present study showed low plasma Ca concentrations and low calcitriol concentrations but a high Ca absorption across the intestinal epithelium, indicating a lack of stimulation of calcitriol synthesis by low plasma Ca levels in goats fed a reduced N and Ca diet.

The mid-jejunal J_{ms} flux rates of mannitol increased in the 7% CP group while the J_{sm} flux rates decreased, resulting in an increase of J_{net} flux rates of mannitol in response to the reduced N and Ca diet. This increase of J_{net} flux rates of mannitol was induced by a greater permeability of the epithelium in mid-jejunum, which might have caused the increase of passive Ca absorption in mid-jejunum of goats fed a reduced N and Ca diet. The reason for the greater permeability of the epithelium for Ca during a reduced protein or reduced N and Ca diet is as yet unknown. The expression level of tight junction proteins like claudin-2 or claudin-12 could be stimulated, both of which function as paracellular Ca channels in single-stomached animals[38].

The increases of the net flux rates of P_i in mid-jejunum during a reduced N diet could not be completely explained by a higher permeability of the epithelium for P_i because only the J_{sm} flux rates of P_i, which were decreased, correlated with the corresponding mannitol fluxes (Fig. 1(b)). The J_{ms} flux rates of P_i increased during the reduction of the dietary N which resulted in an increase of the J_{net} flux rates of P_i. This increase of J_{ms} flux rates of P_i could not be correlated with the corresponding mannitol flux rates (Fig. 1(b)). Therefore, it has to be concluded that the reduction of the dietary N and Ca caused an increase of the active, transcellular Na^+-dependent P_i absorption in mid-jejunum in growing goats.

To investigate the molecular basis of the increase of intestinal net flux rates of Ca and P_i, we performed uptake studies with BBMV from proximal and mid-jejunum to evaluate if the suggested rate-limiting step, the apical uptake of both electrolytes, could be modulated by changes in dietary N and Ca. Actually, the uptake of Ca and P_i into isolated BBMV was not affected at all, in contrast to results of uptake studies from Gaffney-Stomberg *et al.*[9], who showed that a reduction of dietary protein under normocalcaemic conditions caused a decrease of intestinal apical uptake of Ca in rats. The discrepancy between the results performed in Ussing chambers and the data received from uptake studies cannot be explained yet. A possible stimulation of the basolateral located Na^+K^+ATPase could be discussed, which would increase the extrusion of Na^+, generating a higher Na^+ gradient as a driving force for Na^+-dependent transport processes such as P_i or other substrates like glucose in Ussing chamber experiments. Even though apical located Na^+-dependent P_i transporter NaPi IIb was decreased during a reduced N and Ca diet, a hypothetically enhanced Na^+ gradient could potentially compensate for the transporter reduction, which would result in higher intestinal stimulation of Na^+-dependent co-transport systems like P_i and glucose during a reduced N and Ca diet in growing goats.

Furthermore, the mRNA expression of the intestinal Na^+-dependent P_i transporter, NaPi IIb, and the VDR, was not modulated by a reduction of dietary N and Ca in growing goats. Interestingly, the protein expressions of the Ca channel TRPV6 and the P_i transporter NaPi IIb were reduced in the goats in the 7% CP group compared to those animals in the 19% CP group, while the protein expression of the VDR in both epithelia was not affected. The low plasma calcitriol concentrations in the 7% CP could be the reason for the reduction of TRPV6 and NaPi IIb protein expression, assuming that these processes were calcitriol-dependent like in single-stomached animals[14–16].

However, the reduction of protein expression did not verify the higher intestinal net flux rates for Ca and P_i. Besides the

enhancement of the Na^+ gradient by Na^+K^+ATPase, additional Na^+-dependent transporting systems might have been activated during a reduced N and Ca diet in growing goats. A potential candidate could be the L-type Ca channel Ca_v 1·3, which is expressed in the jejunum of rats[39]. The inhibition of Na^+-dependent glucose absorption by phlorizin strongly inhibited intestinal Ca absorption in these rats. Therefore, it was suggested that during a meal, glucose, amino acids and oligopeptide transport caused a depolarisation of the apical membrane, which induced the Ca absorption through L-type Ca channels Ca_v 1·3[39]. Such a depolarisation of the apical membrane was expected during a reduced N and Ca diet in growing goats, too, because a stimulation of the intestinal absorption of glucose could also be observed in these animals (AS Muscher, unpublished results). Therefore, it is hypothesised that this depolarisation could be a reason for the stimulated Ca absorption by the activation of Ca_v 1·3 in proximal jejunum of goats fed a reduced N and Ca diet.

From the present study, it can be concluded that the classical activation of calcitriol by low plasma Ca concentrations does not exist in growing goats fed a reduced N and Ca diet. In contrast to the situation in sheep and goats fed a low-Ca diet only, the intestinal absorption of Ca and P_i was stimulated by this feeding regimen. The involvement of additional transport systems like the L-type Ca channel Ca_v 1·3 and a potential activation of the basolateral located Na^+K^+-ATPase cannot be excluded. Further studies are necessary to characterise the nature of the stimulated electrolyte transport in growing goats fed a reduced N and Ca diet. Additionally, the results of the present study must be evaluated to ascertain whether a reduction of dietary N with adequate Ca levels would also affect intestinal electrolyte absorption in the same manner as seen under hypocalcaemic conditions.

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References

1. Eriksson L & Valtonen M (1982) Renal urea handling in goats fed high and low protein diets. *J Dairy Sci* **65**, 385–389.
2. Silanikove N (1984) Renal excretion of urea in response to changes in nitrogen intake in desert (black Bedouin) and non-desert (Swiss Saanen) goats. *Comp Biochem Physiol A Comp Physiol* **79**, 651–654.
3. Muscher AS, Schroder B, Breves G, *et al.* (2010) Dietary nitrogen reduction enhances urea transport across goat rumen epithelium. *J Anim Sci* **88**, 3390–3398.
4. Harmeyer J & Martens H (1980) Aspects of urea metabolism in ruminants with reference to the goat. *J Dairy Sci* **63**, 1707–1728.
5. Filho JC, Hazel SJ, Anderstam B, *et al.* (1999) Effect of protein intake on plasma and erythrocyte free amino acids and serum IGF-I and IGFBP-1 levels in rats. *Am J Physiol* **277**, E693–E701.
6. Peng YS, Meliza LL, Vavich MG, *et al.* (1974) Changes in food intake and nitrogen metabolism of rats while adapting to a low or high protein diet. *J Nutr* **104**, 1008–1017.
7. Schmidt-Nielsen B (1958) Urea excretion in mammals. *Physiol Rev* **38**, 139–168.
8. Orwoll E, Ware M, Stribrska L, *et al.* (1992) Effects of dietary protein deficiency on mineral metabolism and bone mineral density. *Am J Clin Nutr* **56**, 314–319.
9. Gaffney-Stomberg E, Sun BH, Cucchi CE, *et al.* (2010) The effect of dietary protein on intestinal calcium absorption in rats. *Endocrinology* **151**, 1071–1078.
10. Kerstetter JE, O'Brien KO & Insogna KL (1998) Dietary protein affects intestinal calcium absorption. *Am J Clin Nutr* **68**, 859–865.
11. GfE (2003) *Recommendations for the Supply of Energy and Nutrients to Goats. The Committee for Requirement Standards of the Society of Nutrient Physiology Report* no. 9. Frankfurt am Main: DLG-Verlag.
12. Muscher A & Huber K (2010) Effects of a reduced nitrogen diet on calcitriol levels and calcium metabolism in growing goats. *J Steroid Biochem Mol Biol* **121**, 304–307.
13. Muscher AS, Piechotta M, Breves G, *et al.* (2011) Modulation of electrolyte homeostasis by dietary nitrogen intake in growing goats. *Br J Nutr* **105**, 1619–1626.
14. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, *et al.* (2001) Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci U S A* **98**, 13324–13329.
15. Hattenhauer O, Traebert M, Murer H, *et al.* (1999) Regulation of small intestinal Na-P(i) type IIb cotransporter by dietary phosphate intake. *Am J Physiol* **277**, G756–G762.
16. Yagci A, Werner A, Murer H, *et al.* (1992) Effect of rabbit duodenal mRNA on phosphate transport in *Xenopus laevis* oocytes: dependence on 1,25-dihydroxy-vitamin-D_3. *Pflugers Arch* **422**, 211–216.
17. Ireland P & Fordtran JS (1973) Effect of dietary calcium and age on jejunal calcium absorption in humans studied by intestinal perfusion. *J Clin Invest* **52**, 2672–2681.
18. Frolik CA & DeLuca HF (1972) Metabolism of 1,25-dihydroxycholecalciferol in the rat. *J Clin Invest* **51**, 2900–2906.
19. Naumann C & Basler R (1976) *Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten. Methodenbuch Band III. Die chemische Untersuchung von Futtermitteln. Mit Ergänzungslieferungen 1983, 1993, 1997 (Association of German Agricultural Testing and Research Institutes. Methods Book Volume III. The chemical analysis of feed. With additional deliveries in 1983, 1993, 1997).* Darmstadt: VDLUFA-Verlag.
20. Schröder B, Kappner H, Failing K, *et al.* (1995) Mechanisms of intestinal phosphate transport in small ruminants. *Br J Nutr* **74**, 635–648.
21. Schröder B, Hattenhauer O & Breves G (1998) Phosphate transport in pig proximal small intestines during postnatal

development: lack of modulation by calcitriol. *Endocrinology* **139**, 1500–1507.
22. Auchere D, Tardivel S, Gounelle JC, *et al.* (1998) Role of transcellular pathway in ileal Ca^{2+} absorption: stimulation by low-Ca^{2+} diet. *Am J Physiol* **275**, G951–G956.
23. Schultz SG & Zalusky R (1964) Ion transport in isolated rabbit ileum. I. Short-circuit current and Na fluxes. *J Gen Physiol* **47**, 567–584.
24. Sarkar BC & Chauhan UP (1967) A new method for determining micro quantities of calcium in biological materials. *Anal Biochem* **20**, 155–166.
25. Kruse-Jarres J (1979) *Klinische Chemie, spezielle klinisch-chemische Analytik (Clinical Chemistry, Special Clinical-Chemical Analysis)*. Stuttgart: Fischer-Verlag.
26. Muscher A, Hattendorf J, Pfeffer E, *et al.* (2008) Hormonal regulation of phosphate homeostasis in goats during transition to rumination. *J Comp Physiol B* **178**, 585–596.
27. Schröder B & Breves G (1996) Mechanisms of phosphate uptake into brush-border membrane vesicles from goat jejunum. *J Comp Physiol B* **166**, 230–240.
28. Widiyono I, Huber K, Failing K, *et al.* (1998) Renal phosphate excretion in goats. *Zentralbl Veterinarmed A* **45**, 145–153.
29. Roh SG, Matsunaga N, Miyamoto A, *et al.* (1997) Competitive enzyme immunoassay for bovine growth hormone. *Endocr J* **44**, 195–198.
30. Kawashima C, Sakaguchi M, Suzuki T, *et al.* (2007) Metabolic profiles in ovulatory and anovulatory primiparous dairy cows during the first follicular wave postpartum. *J Reprod Dev* **53**, 113–120.
31. Valtonen MH, Uusi-Rauva A & Eriksson L (1982) The effect of protein deprivation on the validity of creatinine and urea in evaluation of renal function. An experimental study in the goat. *Scand J Clin Lab Invest* **42**, 507–512.
32. Kaune R, Kassianoff I, Schroder B, *et al.* (1992) The effects of 1,25-dihydroxyvitamin D-3 deficiency on Ca(2+)-transport and Ca(2 +)-uptake into brush-border membrane vesicles from pig small intestine. *Biochim Biophys Acta* **1109**, 187–194.
33. Huber K, Walter C, Schroder B, *et al.* (2002) Phosphate transport in the duodenum and jejunum of goats and its adaptation by dietary phosphate and calcium. *Am J Physiol Regul Integr Comp Physiol* **283**, R296–R302.
34. Wilkens MR, Mrochen N, Breves G, *et al.* (2011) Gastrointestinal calcium absorption in sheep is mostly insensitive to an alimentary induced challenge of calcium homeostasis. *Comp Biochem Physiol B Biochem Mol Biol* **158**, 199–207.
35. Schröder B, Kaune R & Harmeyer J (1990) Studies of the porcine intestinal calcitriol receptor in pseudo-vitamin D deficiency rickets type I. *Clin Sci (Lond)* **79**, 409–414.
36. Schröder B, Rittmann I, Pfeffer E, *et al.* (1997) *In vitro* studies on calcium absorption from the gastrointestinal tract in small ruminants. *J Comp Physiol B* **167**, 43–51.
37. Favus MJ, Mangelsdorf DJ, Tembe V, *et al.* (1988) Evidence for *in vivo* upregulation of the intestinal vitamin D receptor during dietary calcium restriction in the rat. *J Clin Invest* **82**, 218–224.
38. Fujita H, Sugimoto K, Inatomi S, *et al.* (2008) Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. *Mol Biol Cell* **19**, 1912–1921.
39. Morgan EL, Mace OJ, Helliwell PA, *et al.* (2003) A role for Ca(v)1·3 in rat intestinal calcium absorption. *Biochem Biophys Res Commun* **312**, 487–493.