

Urinary excretion of purine derivatives as an index of microbial-nitrogen intake in growing rabbits*

J. Balcells†, J. M. Ganuza, J. F. Pérez, S. M. Martín-Orúe and M. González Ronquillo
*Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria,
 Miguel Servet 177, 50013 Zaragoza, Spain*

(Received 17 February 1997 – Revised 14 October 1997 – Accepted 17 October 1997)

Three experiments were carried out to establish a response model between intake and urinary excretion of purine compounds. In Expt 1 the relationship between the intake of purine bases (PB) and the excretion of total purine derivatives (PD) was determined in seven growing rabbits with a mean initial live weight (LW) of 2.03 (SE 0.185) kg, aged 70 d, each fitted with a wooden neck collar to prevent caecotrophagy. They were fed on five experimental diets formulated with different levels of nucleic acids (0.00, 3.75, 7.50, 11.25, 15.00 g yeast-RNA/kg diet). The relationship between intake of purine (x , $\mu\text{mol/kg W}^{0.75}$) and total urinary PD excretion (y , $\mu\text{mol/kg W}^{0.75}$), $y = 0.56 + 0.67x$ (r^2 0.86; RSD 0.338), indicated that about 70% of duodenal PB were recovered as urinary PD and that the endogenous contribution was constant and independent of dietary PB supply. Endogenous excretion of PD (allantoin and uric acid) was measured in a second experiment using six rabbits fed on a purine-free diet and fitted with neck collars to avoid caecotrophagy. Basal daily urinary excretion values for allantoin and uric acid were 532 (SE 33.9) and 55 (SE 7.3) $\mu\text{mol/kg W}^{0.75}$ respectively; xanthine and hypoxanthine were not found in urine samples and therefore the sum of allantoin and uric acid should comprise the total excretion of PD (588 (SE 40.1) $\mu\text{mol/kg W}^{0.75}$). The xanthine oxidase (*EC* 1.2.3.2) activity in plasma, liver, duodenum, jejunum and kidney was measured in a third experiment. The activities of xanthine oxidase in duodenal and jejunal mucosa, liver and kidney were: 0.61 (SE 0.095), 0.37 (SE 0.045), 0.035 (SE 0.001) and 0 units/g fresh tissue respectively and in plasma 2.96 (SE 0.094) units/l. The results show that urinary excretion of PD may be a useful tool to estimate duodenal PB input and microbial protein intake once the relationship between PB and N has been established in caecal micro-organisms.

Purines: Xanthine oxidase: Rabbit

As in ruminants, single-stomached herbivores have developed an especially large colon or caecum to maximize the microbial digestion of fibre. However, while ruminants are able to benefit nutritionally from the microbial mass leaving the rumen, microbial protein in single-stomached herbivores is a waste product contained in faeces. However, some single-stomached species have developed mechanisms to use such material, by combining selective retention in the caecum of fluid and small particles (as vehicles of microbial micro-organisms) with a form of coprophagy: caecotrophagy. For the sake of clarity the term caecotrophagy should be used only in relation to those animals able

to produce two distinct types of faeces of different chemical composition and to consume only one of them: caecotrophes.

In growing rabbits, caecotrophagy may contribute from 15 to 38% of total protein intake (Hörnigke & Björnhag, 1980; Carabaño *et al.* 1988; Fraga *et al.* 1991), this value being affected by several dietary factors such as fibre content (Hörnigke & Björnhag, 1980) and total N or DM intake (Proto *et al.* 1968). Therefore, the caecum in rabbits constitutes a 'fermentation chamber' that can be manipulated in order to optimize the use of dietary protein. The optimization of caecal function has been limited by the

Abbreviations: CMC, carboxymethyl cellulose; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; LW, live weight; PB, purine bases; PD, purine derivatives; PMSF, phenylmethylsulfonyl fluoride; VFA, volatile fatty acids; XO, xanthine oxidase.

*This work was financed by Purina España S.A.

† **Corresponding author:** Dr J. Balcells, fax +34 976 76 16 12, email balcells@posta.unizar.es

experimental difficulties of measuring accurately both production and intake of caecotrophes. Currently, existing methods are based on: (1) the utilization of wooden (or other material) collars to prevent caecotrophy; (2) the establishment of caecum fistulas for the collection of caecal content. However, the use of both methods is restricted to short periods because in long-term experiments the results are always confounded by the fact that caecotrophy is part of the normal physiological digestive process in rabbits. Moreover, it is assumed intrinsically in coprophagy trials that caecotrophes would be fully consumed, even though there is no clear evidence to confirm this (Proto *et al.* 1968). It should also be noted that the use of a collar and fistulation are procedures which normally affect voluntary DM intake.

Purine bases (PB) flowing to the small intestine can be estimated from urinary excretion of purine derivatives (PD; allantoin, uric acid, xanthine and hypoxanthine) once the relationship between duodenal input and urinary excretion of purine compounds is established. This technique has been extensively applied to sheep (Chen *et al.* 1990a; Balcells *et al.* 1991) goats (Lindberg *et al.* 1989) and cows (Verbic *et al.* 1990) to obtain duodenal flow of PB as an index of microbial-N flow. In rabbits and other caecotrophagous animals, the excess of duodenal PB, estimated from urinary excretion of PD, over those ingested in the diet is assumed to come from microbial sources. In these animals, urinary excretion of PD may also constitute a valuable index to estimate microbial protein intake from caecotrophy. The objective of the present study was to establish a response model between duodenal flow and renal excretion of purine compounds in the rabbit, building it on: (1) the relationship between duodenal input and urinary output of purine compounds; (2) the determination of the endogenous contribution to urinary excretion; (3) the activity of the enzyme xanthine oxidase (*EC* 1.2.3.2; XO) in different tissues as a key step for purine metabolism.

Materials and methods

Expt 1: Relationship between purine base intake and urinary excretion of purine derivatives in growing rabbits

Animals. Seven New Zealand White male rabbits with a mean initial live weight (LW) of 2.03 (SE 0.185) kg, aged

70 d, were used. Animals were maintained in metabolism cages for faeces and urine collection, and penned individually during the adaptation period, always under continuous lighting and with free access to drinking water.

Diets. Five experimental diets (Table 1) were formulated based on grass hay (500 g/kg) and barley grain (405 g/kg) supplemented with different amounts of yeast-RNA (Sigma Co., St Louis, MO, USA), (g yeast-RNA/kg DM: 0.00, diet A; 3.75, diet B; 7.50, diet C; 11.25, diet D and 15.00, diet E respectively). Diets were given once daily at a restricted level (50 g/kg LW) to avoid feed refusals. Diets were sampled weekly.

Experimental procedures. Each experimental treatment was maintained for eight consecutive days, comprising 5 d adaptation and 3 d for faeces and urine collection. At 24 h after the start of the experimental period a wooden collar (50 mm i.d. and 270 mm o.d.) was fitted around the neck of each rabbit to avoid caecotrophy, this being removed at the end of each experimental period. Diets were given following a complete 7 × 7 Latin square design where the five experimental diets were completed, along with two additional diets of similar characteristics but in which nucleic acids were supplied as a bacterial concentrate (EA-SACC¹⁰²⁶; Alltech, Inc., Nicholasville, KY, USA) (g EA-SACC/kg DM, 37.5 diet F and 75 diet G). However, analytical determination showed that the purine content in both diets F and G decreased progressively in the weekly samples and therefore data from the EA-SACC diets were removed and are not included in any of the subsequent report or discussions in the present paper. However, diets were supplied following the original schedule. Once the trial was finished, rabbits were fed on the basal diet before they were killed.

Sample collection. Daily urine was collected over H₂SO₄ (100 ml/l, final pH < 3), bulked for each animal and stored at -20°. Faeces were collected after separation of hard and soft material (caecotrophes) and frozen immediately at -20°. After the animals were killed by cervical dislocation, the caecum was excised and weighed. The pH of the caecal content was measured with a glass electrode pH-meter. Three samples of the caecal content were taken, two of them (2 g each) were acidified with either HCl or H₃PO₄, and stored at -20° until the concentrations of NH₃-N and volatile fatty acids (VFA) were determined. The third sample (10 g), obtained for microbial character-

Table 1. Diet composition (g/kg DM) of the experimental diets (A-E) used in Expt 1 and composition of the semi-purified purine-free diet (PF) used in Expt 2

Diet code . . .	A	B	C	D	E	PF
Ingredient						
Grass hay	500	500	500	500	500	0
Barley straw	0	0	0	0	0	554
Barley grain	405	405	405	405	405	0
Starch	0	0	0	0	0	296
Casein	75	71.2	67.5	63.7	60.0	130
Yeast-RNA*	0.00	3.75	7.50	11.25	15.00	0
Vitamin-mineral complex†	20	20	20	20	20	20

* Provided by Sigma Co. (St Louis, MO, USA).

† Provided by Cyanamid Iberica (Madrid, Spain) with the following declared mineral and vitamin composition (g/kg diet): calcium carbonate 2.5; dicalcium phosphate 12.5; sodium chloride 3; Cu 3.5; Zn 7.7; Mn 3.3. Vitamins (mg/kg diet): α -tocopherol 2.2, retinol 540, cholecalciferol 5.

ization, was diluted immediately in anaerobic dilution solution (Bryant & Burkey, 1953) and serially diluted under a stream of CO₂. Portions (1 ml) were inoculated anaerobically in Hungate tubes containing total, cellulolytic or amylolytic culture media. Cellulolytic and amylolytic media were prepared by replacing soluble sugars in the total medium by 30 g/l ball-milled-cellulose (Sigmacell 20, S3504, Sigma) solution and soluble starch respectively. The remainder of the caecal content was diluted in a saline-carboxymethyl cellulose (CMC) solution (9 g NaCl/l, 1 g CMC/l, 38°) and incubated for 5 min at 38° followed by chilling at 4° for 24 h to dislodge adherent bacteria. Diluted caecum content was squeezed through eight layers of surgical gauze and bacteria were isolated from the filtrate by differential centrifugation, 500 g for 5 min, followed by two consecutive centrifugations of the supernatant fraction at 20 000 g for 20 min at 4°. The resultant microbial pellets were freeze-dried for subsequent analysis.

Expt 2. Endogenous excretion of purine derivatives

Animals and feeding. Another set of seven New Zealand White male rabbits (LW 2.93 (SE 0.073) kg) of similar age (70 d) to those used in Expt 1 were given *ad libitum* a semi-purified purine-free diet containing (relative proportion): washed barley straw 0.55, starch 0.30, casein 0.13 and vitamin-mineral mixture 0.02 (Table 1). The semi-purified diet was introduced by gradually replacing a commercial diet for 5 d; after that animals received the purine-free diet for 8 d before the start of the urine collection. Animals were housed and kept under the same conditions as Expt 1.

Excreta collection. The procedures used for the collection of urine and faeces were as described in Expt 1.

Expt 3. Xanthine oxidase activity in tissues and purine compounds in portal and peripheral blood

Animals and sampling. A set of three New Zealand White male rabbits, with a mean LW of 2.88 (SE 0.060) kg, aged 70 d, were used. Animals were fed *ad libitum* during the growth period on a commercial diet (160 g crude protein/kg). Samples of portal and peripheral (heart) plasma were taken by direct extraction from pre-anaesthetized animals and tissue samples were taken after killing the animals by cervical dislocation.

Preparation of tissue extracts. Blood samples were collected into heparinized tubes and centrifuged at 3000 g for 15 min. Plasma samples were analysed within 2 h. Procedures for extraction of liver samples were adapted from those described by Furth-Walker & Amy (1987) and the extraction of the intestinal mucosa layer followed Reeds *et al.* (1997).

Liver and kidney were washed in cold 0.15 M-KCl and 1 g tissue was homogenized in 9 ml 0.5 mM-EDTA in 0.05 M-K₂HPO₄ (pH 7.5) and centrifuged at 35 000 g for 30 min at 4°. The supernatant fraction was dialysed for 24 h against the same EDTA-KH₂PO₄ buffer for 24 h and centrifuged at 35 000 g for 30 min at 4°. The supernatant fraction was used for the assay.

Intestinal samples were taken from the duodenum and jejunum after the Treitz junction and the lumen was washed with cold 0.15 M-KCl. After that intestinal samples were frozen immediately (using liquid N) and defrosted within 2 h at 4°, then the lumen was washed with 0.05 M-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.5) containing 0.25 mM-EDTA and 0.25 mM-phenylmethylsulfonyl fluoride (PMSF). A sample of intestinal mucosa (1 g) was removed by finger pressure along the portion of intestine, collecting the mucosa layer cells at the bottom in 9 ml HEPES-EDTA-PMSF buffer. The extract containing the enzyme XO was then purified as for the liver samples but using HEPES-EDTA-PMSF buffer.

Xanthine oxidase activity. Activity of XO was measured as the rate of uric acid production when xanthine was incubated with tissue extracts as described by Chen *et al.* (1996).

Chemical analyses

Urinary and plasma PD (allantoin, uric acid, hypoxanthine and xanthine) were analysed by reverse-phase HPLC, using two Spherisorb C-18 ODS-2 (4.6 × 250 mm) columns, according to the technique described by Balcells *et al.* (1992). After defrosting, a significant amount of mineral precipitation appeared in the jar containing urine. The precipitate was rediluted by shaking it with 100 ml buffer (0.2 M-phosphate-glycerol; 90:10, v/v, pH 8-9) for 30 min and the resultant dilution was treated in the same way as urine samples. The values obtained were added to the urinary ones. Adenine and guanine in feeds (50 mg), caecal bacteria (15 mg), caecotrophes (15 mg), and hard faeces (100 mg) were determined by the same HPLC technique, after their hydrolysis with 2 ml 2 M-HClO₄ at 100° for 1 h, adding previously 0.20 μmol allopurinol as an internal standard and neutralizing immediately with 4.5 M-KOH (Martín-Orúe *et al.* 1995). DM was determined by drying at 105° to constant weight. N was measured by the Kjeldahl method. Neutral-detergent fibre and acid-detergent fibre were determined according to the procedure described by Van Soest *et al.* (1991) using an amylolytic pre-treatment. VFA were analysed by GLC following the procedure described by Jouany (1982) and caecum NH₃ using the method proposed by Chaney & Marbach (1962).

Statistical analysis

Values from Expt 1 were examined by ANOVA as an incompletely balanced block design (Youden square) where five treatments were tested against seven animals during seven experimental periods. Analyses were performed following the procedure described by Box *et al.* (1978). Treatment sums of squares were partitioned into two orthogonal contrasts to test linear and quadratic responses to PB supplementation. Regression analysis was also used to describe this response (Steel & Torrie, 1980). In the third experiment uric acid production was calculated using a standard curve established with uric acid and described linearly in the liver and as a monoexponential

function of the incubation time ($y = a + b(1 - e^{-ct})$) as described by Chen *et al.* (1996) in the intestinal mucosa and plasma. One unit of XO activity was defined as 1 μmol uric acid produced per min at 37° in excess of substrate.

Results

Feed intake was restricted in Expt 1 to 50 g/kg LW per d in order to avoid feed refusals and to maintain a stable supply of nutrients. In such conditions, animals increased LW from 2.03 (SE 0.078) kg (70 d, initial LW) to 2.96 (SE 0.062) kg (128 d, final LW) so they showed a moderate LW gain rate (16 g/d) which was not different among experimental periods ($P > 0.10$). In Expt 2, animals remained in good health throughout the experiment, but one animal failed to maintain an acceptable level of DM intake and the corresponding observations of endogenous excretion were removed. Animals could not meet their maintenance requirements and they lost weight, but the loss was less than 0.7 % of the initial body weight.

Caecum characteristics

The average NH_3 concentration in caecal content was 9.3 (SE 0.93) mmol/l and the pH was constant among animals with a mean value of 6.4 (SE 0.08). The total concentration of VFA in caecal content was 73 (SE 3.2) mmol/l with acetate comprising 80.5 % of the total followed by butyrate (9.4 %) and propionate (5.9 %). The total count of micro-organisms in the caecum was 10^9 micro-organisms/ml with the highest proportion being amylolytic micro-organisms (12×10^6) whereas the count of cellulolytic organisms was only 23×10^4 micro-organisms/ml. Fungi and protozoa were not detected in caecal contents.

Chemical composition of isolated bacteria, caecotrophes, and hard faeces and estimation of recycled nitrogen

Fig. 1 illustrates the chemical composition (N, PB and organic matter) of caecum bacterial extracts, soft and hard faeces from animals eating the basal diet (A). Compared with hard faeces, soft faeces showed a higher content of total N (45.9 v. 16.5 g/kg DM), PB (47.7 v. 8.25 mmol/kg DM) and PB:N (1.03 v. 0.45 mmol/g). There were no differences in organic matter content between the two substrates ($P > 0.1$). Isolated bacteria showed higher contents of PB (94.6 mmol/kg DM) and N (66.4 g/kg DM) than soft faeces, but differences in PB were higher than those in N content, explaining the lower PB:N ratio in soft faeces (1.03 (SE 0.083) mmol/g) than those values obtained in microbes (1.42 (SE 0.128) mmol/g).

Among diets there were no differences ($P > 0.1$) in either DM digestibility (51.6 (SE 1.06) %) or faecal output of both hard (41.4 (SE 1.26) g/d) and soft faeces (11.01 (SE 0.92) g/d). Using PB as a microbial marker, microbial N excretion in soft faeces was higher (0.357 (SE 0.011) g/d) than in hard faeces (0.230 (SE 0.007) g/d) and, assuming that soft faeces were fully consumed, microbial N intake would be about 60 % of total microbial N synthesized in the caecum.

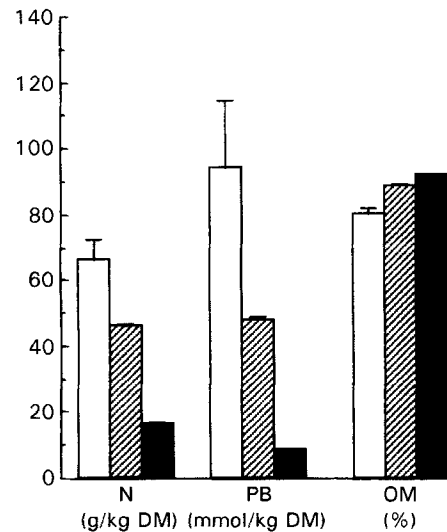


Fig. 1. Nitrogen (g/kg DM), purine bases (PB; mmol/kg DM) and organic matter (OM; %) content of caecum bacterial extract (□), soft faeces (▨) and hard faeces (■) from rabbits fed on a basal diet (diet A). For details of diets, see Table 1. Values are means for seven animals, with standard deviations indicated by vertical bars.

Endogenous excretion of purine derivatives

The results from animals eating a purine-free diet are presented in Table 2. Adenine and guanine were not found in urine samples and only trace amounts of xanthine plus hypoxanthine were detected, thus the sum of allantoin and uric acid should comprise all the endogenous purine compounds excreted in urine. Total endogenous excretion of PD in six male rabbits was 588 (SE 40.1) $\mu\text{mol/kg W}^{0.75}$ per d, with relative proportions of allantoin and uric acid being 0.9 and 0.1 respectively. The between-animal variation (CV) of endogenous excretion was 6.8%. Creatinine excretion during endogenous trial averaged 2.5 (SE 0.32) mmol/d, slightly lower than values obtained in Expt 1 (2.63 to 2.80 mmol/d) but differences did not reach statistical significance.

Tissue xanthine oxidase activity and purine derivative appearance in portal and peripheral blood

With the reservation that the number of animals was small, Fig. 2 shows the increase in uric acid when xanthine was

Table 2. Expt 2. The endogenous excretion of urinary purine derivatives (PD; $\mu\text{mol/kg W}^{0.75}$) by non-caecotrophagous growing rabbits fed on a semi-purified purine-free diet*

Animal	LW (kg)	Uric acid	Allantoin	Total PD
R ₁	2.74	38.3	419	458
R ₂	2.78	40.9	558	599
R ₄	2.99	43.1	488	531
R ₅	2.96	75.0	627	703
R ₆	3.24	79.4	620	699
R ₇	2.86	54.9	483	538
Mean		55	532	588
SE		7.33	33.9	40.1

LW, live weight.

* For details of diets and procedures, see Table 1 and pp. 374–375.

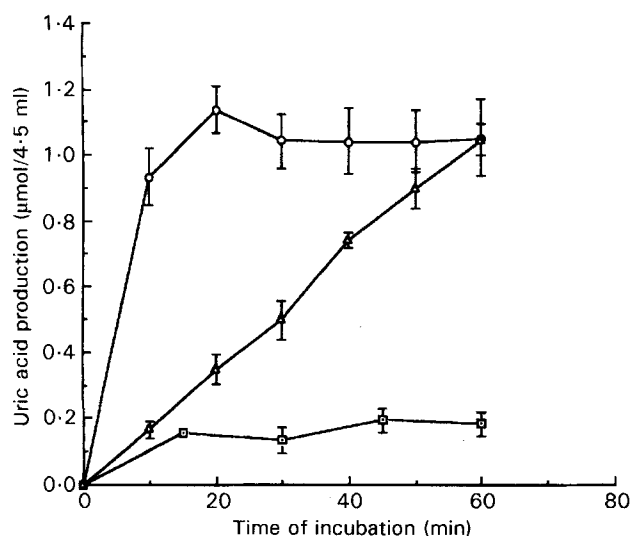


Fig. 2. Uric acid production from xanthine incubated with plasma (\square), liver extracts (Δ) or intestinal mucosa (\circ) from growing rabbits. Values are means for three animals, with standard deviations indicated by vertical bars. For details of procedures, see pp. 374–375.

Table 3. Expt 3. Activities* of xanthine oxidase (EC 1.2.3.2; XO) in plasma, liver and intestinal mucosa of growing rabbits

Animals	LW (kg)	Plasma (units/l)	Liver (units/g)	Duodenum (units/g)	Jejunum (units/g)
A	2.7	0.0057	0.037	0.696	0.448
B	3.1	0.0012	0.037	0.759	0.404
C	2.9	0.0035	0.032	0.381	0.265
Mean	2.96	0.0035	0.036	0.612	0.37
SE	0.094	0.00107	0.0013	0.0954	0.045

LW, live weight.

* One unit of XO activity was defined as 1 μmol uric acid produced per min at 37° with excess substrate.

incubated with different tissue extracts as a measure of their XO activity. Estimated values of XO activity (units/min) in plasma, intestine and liver are presented in Table 3. The XO activity was much higher in intestine than in liver whereas plasma showed only traces of XO activity. Enzymic activity decreased along the intestine, showing a lower value in the jejunum (0.36 (SE 0.045) units/min) than in the duodenum (0.61 (SE 0.095) units/min). No XO activity was detected in kidney extracts. Table 4 illustrates the PD appearance in portal and peripheral (heart) plasma.

Table 4. Expt 3. Concentration of purine derivatives ($\mu\text{mol/l}$) in portal and peripheral blood of three rabbits

Rabbit	Allantoin		Uric acid		Hypoxanthine		Xanthine		Creatinine	
	Portal	Heart	Portal	Heart	Portal	Heart	Portal	Heart	Portal	Heart
A	91.0	91.4	48.2	30.0	20.9	8.1	9.97	3.2	87.2	90.6
B	89.3	80.4	35.1	26.8	12.2	5.1	4.8	ND	78.2	71.4
C	54.2	49.4	8.4	14.2	170.5	15.6	35.9	5.9	57.5	58.3
Mean	78.2	73.7	30.6	23.7	67.9	9.6	16.9	4.6	74.3	73.4
SE	11.99	12.57	11.71	4.82	51.37	3.12	9.61	1.70	8.79	9.37

ND, not detected.

Allantoin concentration in normally fed rabbits was similar in both vessels (78.2 v. 73.7 $\mu\text{mol/l}$) although allantoin precursors did show a much lower concentration in peripheral than in portal blood ($P < 0.01$). On average peripheral heart blood contained 77 % of the uric acid, 14 % of the hypoxanthine and 26 % of the xanthine present in the portal vessel. Creatinine concentrations in the two vessels were similar (74.3 and 73.4 $\mu\text{mol/l}$ for portal and peripheral blood samples).

Urinary excretion of purine derivatives at different levels of purine base intake

Urinary excretion of total PD responded rapidly to changes in the level of PB intake (Table 5). The main increase in PD excretion, associated with dietary PB supply, was allantoin (from 3.2 to 6.2 mmol/d) although uric acid excretion showed a lower but significant increase (from 0.3 to 0.6 mmol/d). As in the endogenous trial (Expt 2), xanthine and hypoxanthine excretion in urine was negligible. The proportions of allantoin and uric acid in urine samples were constant throughout the experimental period, allantoin representing 90 % of total PD excretion.

PD excretion responds linearly to PB input although allantoin ($P < 0.1$) and total PD ($P < 0.05$) reached a maximum at the fourth level of PB supply (diet D) and therefore the quadratic component of the response also reached statistical significance ($P < 0.01$) for both compounds (Table 5).

Creatinine excretion was not affected by the experimental treatment and averaged 1.36 (SE 0.097) mmol/kg $W^{0.75}$. The ratio total PD:creatinine concentration (mol/mol) in the urine increased linearly from 1.19 to 2.50.

Discussion

Soft faeces production and caecal fermentation variables

Faecal excretion of soft faeces obtained in the present work (11.0 g/d) were lower than values reported by Gidenne & Lebas (1987; 24.2 g/d) and Carabaño *et al.* (1988; 15–29.6 g/d) but similar to those reported by Fraga *et al.* (1991; 7.5–11.5 g/d). It is necessary to remark that most of these values correspond to a period of collection of 20–24 h whereas our values were obtained throughout a period of 72 h. Changes in soft faeces excretion have been related to the collection procedure (Bellier & Gidenne, 1996), age of rabbit (Gidenne & Lebas, 1987) and diet

Table 5. Expt 1. Urinary excretion of purine derivatives (PD; allantoin and uric acid) and creatinine from male non-caecotrophagous rabbits fed on a grass hay–barley diet supplemented with increasing doses of nucleic acids from yeast-RNA†

(Mean values with their pooled standard error for seven rabbits)

Diet code ...	A	B	C	D	E	SE	Statistical significance of effect of PB supplementation	
							Lin	Quad
PB intake (mmol/d)	2.32	4.70	6.50	8.48	10.30	0.301	***	NS
Urinary excretion								
Allantoin (mmol/d)	3.18	4.17	5.31	6.10	6.21	0.217	***	**
Uric acid (mmol/d)	0.31	0.38	0.36	0.62	0.60	0.082	**	NS
Total PD (mmol/d)	3.34	4.55	5.67	6.71	6.80	0.300	***	*
(mmol/kg W ^{0.75})	1.62	2.21	2.79	3.33	3.36	0.191	***	NS
Creatinine (mmol/d)	2.76	2.63	2.92	2.80	2.72	0.240	NS	NS
(mmol/kg W ^{0.75})	1.36	1.29	1.43	1.37	1.36	0.097	NS	NS
Allantoin/creatinine	1.19	1.73	2.01	2.57	2.50	0.237	***	***

Lin, linear; Quad, quadratic; PB, purine bases.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

† For details of diets and procedures, see Table 1 and pp. 374–375.

characteristics (Carabaño *et al.* 1988). Thus, our experimental procedures, which required prevention of caecotrophy during a period of 6 d before soft faeces collection, may have affected to some extent the daily excretion of soft faeces. In any case, the fermentation variables in caecal content (pH and VFA content) were similar to those reported by other workers (Jehl & Gidenne, 1996) and the NH₃ concentration in particular was also in the range described in the literature (Carabaño *et al.* 1988; Fraga *et al.* 1991; Jehl & Gidenne, 1996) and adequate for optimal microbial synthesis (5–15 mg/100 ml; Balcells *et al.* 1993).

A single microbial isolate was obtained from a mixed sample of the whole caecal population. The chemical composition of this isolate was within the range reported in the literature for rumen micro-organisms (Legay-Carmier & Bauchart, 1989; Pérez *et al.* 1996), but closer to the values described for an adherent population than for liquid-associated bacteria (Pérez *et al.* 1996). Assuming that dietary PB are mostly digested in the small intestine (Storm *et al.* 1983) or are quickly degraded by caecal fermentation (Surra *et al.* 1997), PB in faeces can be considered to be of microbial origin, and used as a microbial marker. Thus, the higher concentration of PB in soft than in hard faeces confirms the high efficiency of the large intestine in rabbits to concentrate the caecum microbial yield in the soft faecal material, allowing recycling of microbial-N through caecotrophy. In spite of this, about 40% of the caecal net microbial synthesis (soft + hard faeces PB) was lost throughout the excretion of hard faeces.

Endogenous excretion of purine derivatives

Although the animals lost weight during the experiment, no other apparent disturbances were observed. The endogenous excretion observed in the present study was similar (588 µmol/kg W^{0.75}) to those values reported in cattle (500–514 µmol/kg W^{0.75}) by Chen *et al.* (1990b) and Giesecke *et al.* (1993), but much higher than those measured in sheep (165–202 µmol/kg W^{0.75}; Chen *et al.* 1990b; Balcells *et al.* 1991), pigs (166 and 199 µmol/kg

W^{0.75}; Chen *et al.* 1990b; Martín-Orúe *et al.* 1995) and man (70–80 µmol/kg W^{0.75}, assuming 70 kg LW; Zollner, 1982). Higher values were reported by Greife (1980) in rats (970 µmol/kg W^{0.75}). Species differences in the urinary endogenous excretion of PD were also evident in the form of the derivatives excreted. In sheep and pigs re-usable-PD (xanthine plus hypoxanthine) accounted for a considerable proportion of total PD (Chen *et al.* 1990b; Balcells *et al.* 1991); whereas in cattle, buffalo (Chen *et al.* 1996) and rabbits both compounds were negligible. Changes in the magnitude and profile of the endogenous PD have been related to differences in the tissue XO activity.

Tissue xanthine oxidase activities and blood concentration of purine derivatives

The present study in rabbits showed differences in the level of XO activity in plasma, liver and intestine, and also a decreasing level of activity between duodenum and jejunum (Table 3); XO activity was negligible in kidney. PD concentrations in plasma samples were higher than values reported previously by Al-Khalidi & Chaglassian (1965) but these differences could be explained by the modification of the analytical procedure consisting of the inclusion of L-histidine to prevent inhibition of XO by excess substrate (Chen *et al.* 1996). The authors are unaware of additional data on tissue XO activity in rabbit tissues, but present values are similar to those reported in the rat. Mohamedali *et al.* (1993) reported a much higher XO activity in rat duodenum (3.29 units/g) than in liver (about 0.20 units/g) whereas only traces of XO activity were detected in kidney. These authors also found that enzyme activity was highest in the duodenum and decreased across the small intestine with only trace amounts being found in hindgut.

Among species, Chen *et al.* (1996) reported a higher XO activity in cattle or buffalo than in sheep. Whereas sheep showed only trace amounts of enzyme, buffalo and cattle had high levels of XO activity in liver (0.44 and 0.30 units/g), intestinal mucosa (0.31 and 0.18 units/g)

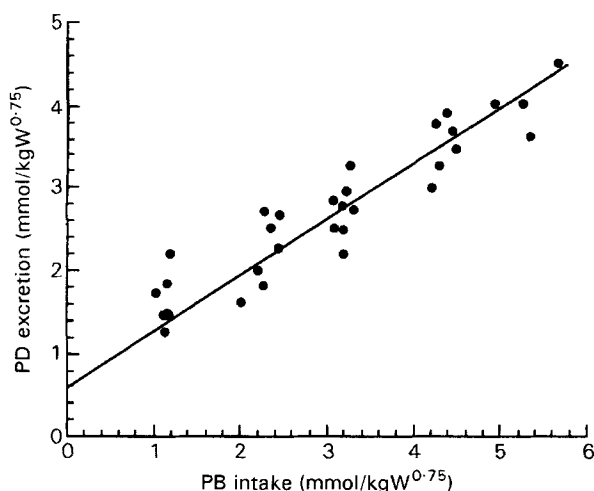


Fig. 3. Urinary excretion of purine derivatives (PD) in relation to intake of purine bases (PB) in rabbits ($\text{mmol/kg W}^{0.75}$). The relationship is described by the equation $y = 0.59 + 0.67x$; R^2 0.86, n 32.

and plasma (24.5 and 1.4 units/l, respectively). In rabbits the high XO activity determined in intestinal mucosa suggests that dietary nucleic acid bases are rapidly degraded to oxidized derivatives across the intestinal mucosa and hepatic metabolism, but small significant amounts of reusable PD (xanthine plus hypoxanthine; Table 4) were still detected in peripheral blood.

Relationship between excretion of purine derivatives and purine intake

Three animals showed a biased urinary recovery of ingested PB when they were fed on the highest level of PB intake (diet E). These animals received the higher levels of dietary PB supply during the earlier periods of the experiment. Because of the high level of dietary PB supply and the significant changes in intestinal structure and function with rabbit ageing (Thomson, 1986), these values were removed from all calculations. In any case, when data from these animals were included, both intercept and incremental recovery were not significantly different to the presented equation, but the correlation was lower (r^2 0.699).

Daily excretion of total PD (y ; $\text{mmol/kg W}^{0.75}$) was correlated linearly with intake of total PB (x ; $\text{mmol/kg W}^{0.75}$), since the inclusion of a curvilinear component did not significantly improve the adjustment. It was assumed that the intercept ($x=0$) was equal to endogenous excretion (0.588 $\text{mmol/kg W}^{0.75}$). The resultant equation was:

$$y = 0.59 \text{ (SE } 0.136) + 0.67 \text{ (SE } 0.018) x$$

$$(r^2 \text{ } 0.860, \text{ RSD } 0.338, n \text{ } 32; \text{ Fig. } 3).$$

Incremental recovery of ingested (0.67) or absorbed PB (0.73), assuming the digestibility ratio 0.91 (Chen *et al.* 1990a), indicates that absorbed PB are not fully recovered in the urine and a significant fraction may be lost through other routes, for example saliva (Chen *et al.* 1990a; Surra *et al.* 1997) or through the hindgut (Berlin & Hawkins, 1968).

A linear model to describe the relationship between urinary excretion and intake of purine compounds has been used in man (Zollner, 1982), other single-stomached animals (Giesecke & Timeyer, 1982) and cattle (Verbic *et al.* 1990). This previous response model assumes that there is a constant endogenous excretion of PD coming from *de novo* synthesis processes defining the balance between salvage pathway and *de novo* synthesis at a cellular level. Thus it has been suggested that XO activity in the different tissues not only degrades purines to non-recoverable PD but also regulates the balance between degradation and salvage of purine compounds (Kaminski & Jezewska, 1979). Rabbits showed a similar XO profile to those seen in rat, man and cattle, and then a similar model to those described in these species would be expected.

If the urinary excretion of PD is a linear function of duodenal flow of PB and it is assumed that PB-digestibilities of microbial and dietary sources are equal, microbial-N intake from caecotrophy can be estimated by subtracting those purines ingested with feed from the duodenal PB-flow. The following equation based on the response model (Fig. 3) and the PB:N ratio obtained in caecal flora (1.42 mmol/g) is suggested:

$$\text{microbial-N intake (g/kg W}^{0.75}) = 1.05 y_1 - 0.70 x_2 - 0.6,$$

where y_1 is PD excreted in urine ($\text{mmol/kg W}^{0.75}$) and x_2 is dietary intake of PB ($\text{mmol/kg W}^{0.75}$).

The precision of the reported relationship between purine intake and urinary excretion of PD and the significant contribution of microbial PB to the total purine intake support this method as a simple and useful tool to estimate, under practical conditions, the microbial protein intake of growing rabbits.

Acknowledgements

The authors wish to thank J. A. Guada and M. Fondevila for the critical review of this manuscript.

References

- Al-Khalidi UAS & Chaglassian TH (1965) The species distribution of xanthine oxidase. *Biochemical Journal* **97**, 318–320.
- Balcells J, Fondevila M, Guada JA, Castrillo C & Surra JC (1993) Urinary excretions of purine derivatives and nitrogen in sheep given straw supplemented with different sources of carbohydrates. *Animal Production* **57**, 287–292.
- Balcells J, Guada JA, Castrillo C & Gasa J (1991) Urinary excretion of allantoin and allantoin precursors by sheep after different rates of purine infusion into the duodenum. *Journal of Agricultural Science, Cambridge* **116**, 309–317.
- Balcells J, Guada JA, Peiró JM & Parker DS (1992) Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. *Journal of Chromatography* **575**, 153–157.
- Bellier R & Gidenne T (1996) Consequences of reduced fibre intake on digestion, rate of passage and caecal microbial activity in the young rabbit. *British Journal of Nutrition* **75**, 353–363.
- Berlin RD & Hawkins RA (1968) Secretion of purines by the small intestine: general characteristics. *American Journal of Physiology*, **215** 932–941.

- Box GEP, Hunter WG & Hunter JS (1978) *Statistics for Experimenters. An Introduction to Design, Data Analysis and Model Building*. New York: J. Wiley & Sons, Inc.
- Bryant MP & Burkey LAC (1953) Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *Journal of Dairy Science* **36**, 205–217.
- Carabaño R, Fraga MJ, Santoma G & de Blas C (1988) Effect of diet on composition of cecal contents and on excretion and composition of soft and hard feces of rabbits. *Journal of Animal Science* **66**, 901–910.
- Chaney AL & Marbach EP (1962) Modified reagents for determination of urea and ammonia. *Clinical Chemistry* **8**, 130–132.
- Chen XB, Hovell FDeB, Ørskov ER & Brown DS (1990) Excretion of purine derivatives by ruminants: effect of exogenous nucleic acid supply on purine derivative excretion by sheep. *British Journal of Nutrition* **63**, 131–142.
- Chen XB, Ørskov ER & Hovell FDeB (1990) Excretion of purine derivatives by ruminants: endogenous excretion, differences between cattle and sheep. *British Journal of Nutrition* **63**, 121–129.
- Chen XB, Samarawera L, Kyle DJ, Ørskov ER & Abeygunawardene H (1996) Urinary excretion of purine derivatives and tissue xanthine oxidase (EC 1.2.3.2) activity in buffaloes (*Bubalis bubalis*) with special reference to differences between buffaloes and *Bos taurus* cattle. *British Journal of Nutrition* **75**, 397–407.
- Fraga MJ, Pérez de Ayala P, Carabaño R & de Blas JC (1991) Effect of type of fiber on the rate of passage and on the contribution of soft feces to nutrient intake of finishing rabbits. *Journal of Animal Science* **69**, 1566–1574.
- Furth-Walker D & Amy NK (1987) Regulation of xanthine oxidase activity and immunologically detectable protein in rats in response to dietary protein and iron. *Journal of Nutrition* **117**, 1697–1705.
- Gidenne T & Lebas F (1987) Estimation quantitative de la caecotrophie chez le lapin en croissance: variations en fonction de l'âge (Quantitative estimation of the caecotrophy in growing rabbits: age function variations). *Annales de Zootechnie* **36**, 225–236.
- Giesecke D, Balsliemker J, Südekum KH & Stangassinger M (1993) Plasma level, clearance and renal excretion of endogenous and ruminal purines in the bovine. *Journal of Animal Physiology and Animal Nutrition* **70**, 180–189.
- Giesecke D & Tiemeyer W (1982) Availability and metabolism of purines of single-cell proteins in monogastric animals. *Proceedings of the Nutrition Society* **41**, 319–327.
- Greife HA (1980) Nitrogen utilization of microbial nucleic acids in the growing rat. In *Proceedings of the 3rd EAAP Symposium on Protein Metabolism and Nutrition, Braunschweig, F.R. Germany, May 1980*. European Association of Animal Production, Publication no. 27, pp. 159–167 [HJ Oslage and K Rohr, editors]. Braunschweig: Institute of Animal Production.
- Hörnigle H & Björnhag G (1980) Coprophagy and related strategies for digesta utilization. In *Digestive Physiology and Metabolism in Ruminants*, p. 708 [Y. Ruckebusch and P. Thivend editors]. Lancaster: MTP Press.
- Jehl N & Gidenne T (1996) Replacement of starch by digestible fibre in feed for the growing rabbit 2. Consequences for microbial activity in the caecum and on incidence for digestive disorders. *Animal Feed Science and Technology* **61**, 193–204.
- Jouany JP (1982) Volatile fatty acid and alcohol determinations in digestive contents, silage juices, bacterial cultures and anaerobic fermentors contents. *Science des Aliments* **2**, 131–144.
- Kaminski ZW & Jezeswka MM (1979) Intermediate dehydrogenase-oxidase form of xanthine oxidoreductase in rat liver. *Biochemical Journal* **181**, 177–182.
- Lindberg JE, Bristav H & Manyenga AR (1989) Excretion of purines in the urine of sheep in relation to duodenal flow of microbial protein. *Swedish Journal of Agricultural Research* **19**, 45–52.
- Legay-Carmier F & Bauchart D (1989) Distribution of bacteria in the rumen contents of dairy cows given a diet supplemented with soya-bean oil. *British Journal of Nutrition* **61**, 725–740.
- Martín-Orúe SM, Balcells J, Guada JA & Castrillo C (1995) Endogenous purine and pyrimidine derivative excretion in pregnant sows. *British Journal of Nutrition* **73**, 375–385.
- Mohamedali KA, Guicherit OM, Kellems RE & Rudolph FB (1993) The highest levels of purine catabolic enzymes in mice are present in the proximal small intestine. *Journal of Biological Chemistry* **268**, 23728–23733.
- Pérez JF, Balcells J, Guada JA & Castrillo C (1996) Determinations of rumen microbial-N production in sheep. A comparison of urinary purine excretion with methods using ¹⁵N and purine bases as markers of microbial-N entering the duodenum. *British Journal of Nutrition* **75**, 699–709.
- Proto V, Gargano D & Gianani L (1968) La coprofagia del coniglio sotto posto a differenti diete (Coprophagy in the rabbit under different diets). *Produccion Animal* **7**, 157.
- Reeds PJ, Burrin DG, Stoll B, Jahoor F, Wykes I, Henry J & Frazer M (1997) Enteral glutamate is the preferential source for mucosal glutathione synthesis in fed piglets. *American Journal of Physiology* **273**, 408–415.
- Steel RGD & Torrie JH (1980) *Principles and Procedures of Statistics*, 2nd ed. New York: McGraw-Hill.
- Storm E, Brown DS & Ørskov ER (1983) The nutritive value of rumen micro-organisms in ruminants. 3. The digestion of microbial amino and nucleic acids in, and losses of endogenous nitrogen from, the small intestine of sheep. *British Journal of Nutrition* **50**, 479–485.
- Surra JC, Guada JA, Balcells J & Castrillo C (1997) Effects of post ruminal fermentation of the faecal and urinary excretion of purines. *Animal Science* **65**, 383–390.
- Thomson AB (1986) Early nutrition and intestinal transport function: effect of low-cholesterol diet. *Journal of Laboratory and Clinical Medicine* **107**, 365–377.
- Van Soest PJ, Robertson JB & Lewis RA (1991) Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74**, 3583–3597.
- Verbic J, Chen XB, Macleod NA & Ørskov ER (1990) Effect of microbial nucleic acid infusion on purine derivative excretion by steers. *Journal of Agricultural Science, Cambridge*, **114**, 243–248.
- Zollner N (1982) Purine and pyrimidine metabolism. *Proceedings of the Nutrition Society* **41**, 329–342.