Nitrogen excretion in germ-free and conventional chickens: effects of an alkali load

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1. Groups of three colostomized germ-free (GF) and conventional (CV) chickens aged 4 months were maintained for successive periods of 8 d on a diet containing 200 g casein/kg without and with sodium bicarbonate at the rate of 20 mmol/d and a nitrogen-free diet without and with $NaHCO_3$ at 9 mmol/d. Urine and faeces were collected during the last 3 d of each period.

2. Total N, uric acid- and ammonia-N were determined in urine and total N in faeces. Amino acids were measured in hydrolysates of faeces collected during the periods when no $NaHCO_3$ was included in the diets.

3. The CV birds excreted more N on the casein diets but less on the N-free diets than did their GF counterparts, the differences being mainly shown in the urine.

4. On both diets hydrolysates of the faeces of CV birds contained smaller amounts of amino acids. On the N-free diet the proportions (g/160 g N) of serine, proline and threonine were reduced, suggesting some conservation of endogenous N by micro-organisms, and the proportions of histidine, alanine, lysine and methionine increased, possibly through microbial synthesis; on the casein diet, proportions of most amino acids were less, probably because bacterial deamination had occurred.

5. Urinary excretion of total N, uric acid and ammonia was much greater on the casein than on the N-free diets. Inclusion of $NaHCO_3$ caused a sharp fall in urinary ammonia on both diets and in both environments.

6. It was concluded that the level of dietary protein and the regulation of acid-base balance have more effect than microbial activity on the urinary ammonia excretion.

Urinary ammonia in the chicken arises mainly as an end-product of catabolism of protein. Its concentration depends upon the amount of protein in the diet (Tasaki & Okumura, 1964) and also upon the acid-base balance of the body (Okumura & Tasaki, 1968). Thus it appears to have two functions, one as a means of eliminating excess nitrogen and the other as an acid-base balance regulator. The activities of the gut microflora may also influence urinary ammonia excretion which, if increased, would partly account for the greater endogenous N loss in the combined urine and faeces of germ-free compared with conventional chicks (Miller, 1967; Salter, Coates & Hewitt, 1974). The experiments reported here were done with colostomized chickens so that N excretion could be measured separately in urine and faeces. They were designed to compare N excretion in germ-free and conventional environments by chickens given diets adequate in protein or N free, and to determine the effects under all these conditions of an alkali load.

MATERIALS AND METHODS

Germ-free (GF) chickens of the Rhode Island Red × Light Sussex cross were reared from hatching to 3 months of age in large Gustafsson stainless-steel isolators as described by Coates, Fuller, Harrison, Lev & Suffolk (1963). Conventional (CV) birds from the same hatch of eggs were reared in a clean but not sterile environment. In order to collect urine and faeces separately an artificial anus was surgically produced in the birds by the method of Ariyoshi & Morimoto (1956), as modified by Okumura (1976). This enabled quantitative collection of faeces and urine in plastic bags through funnels attached to the artificial and natural

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anuses respectively. The birds were kept in individual metabolism cages and fed on a practical chick mash for about 1 month after the operation so that they recovered fully from the effects of surgery and became accustomed to the collection procedure. There were two pullets and one cock in the germ-free isolators and three pullets in the conventional environment.

Diets

The composition (g/kg) of the N-free diet was: maize starch 815·4, maize oil 80, cellulose powder 25, methyl cellulose 10, salt mixture 60, vitamin supplement 8·1, choline chloride 1·5. In each kg diet the salt mixture provided $CaCO_3$ 17·1 g, KH_2PO_4 13·3 g, $CaHPO_4.2H_2O$ 17·1 g, NaCl 8·67 g, $MgSO_4.H_2O$ 2·67 g, $MnSO_4.4H_2O$ 270 mg, KI 37 mg, $CuSO_4.5H_2O$ 16 mg, $ZnSO_4.7H_2O$ 130 mg, $FeSO_4.7H_2O$ 670 mg. Fat-soluble vitamins were dissolved in the maize oil to provide 0·16 mg cholecalciferol, 20 mg menaphthone and 40 mg α-tocopheryl acetate in each kg diet. Rovimix A500 (Roche Products, Welwyn Garden City, Herts) was added to supply 20 mg retinol/kg. The vitamin triturate provided (/kg diet): calcium pantothenate 60 mg, riboflavin 24 mg, thiamin hydrochloride 12 mg, pyridoxine hydrochloride 16 mg, nicotinic acid 160 mg, pteroylmonoglutamic acid 6 mg, biotin 800 μg, cyanocobalamin 80 μg. In the casein diet an appropriate amount of the maize starch was replaced by 200 g casein and 1·6 g L-methionine/kg. Where an alkali load was necessary $NaHCO_3$ was added at the expense of maize starch to a concentration of 250 mmol/kg in the casein diet and 112·5 mmol/kg in the N-free diet (to provide 20 and 9 mmol/d respectively). The diets were granulated after mixing, methyl cellulose providing the binding agent. They were packed in plastic bags, sealed and sterilized by γ-radiation at 5 Mrad.

Analytical methods

Total N of urine and faeces was determined by semi-micro Kjeldahl digestion followed by measurement of the ammonium sulphate formed in a Technicon Autoanalyser (Technicon Instruments Co. Ltd, Chertsey, Surrey). The concentration of uric acid was determined by a uricase method using the Technicon Autoanalyser (Morgenstern, Flor, Kaufman & Klein, 1966). Conway's method (Conway, 1957) was used to determine ammonia-N. Faecal amino acids were measured in hydrolysates prepared by refluxing samples containing about 50 μg N with 6 M-HCl for 24 h in an oil bath at 110°, concentrating by rotary evaporation at 45° and redissolving in 0·11 M-sodium citrate buffer at pH 2·2. For determinations of cystine and methionine, a sample was treated by performic acid oxidation (Moore, 1963) before undergoing hydrolysis. Measurements were made in a JEOL Amino Acid Analyser (Model JLC-5AH; Japan Electric Optics Laboratory Co. Ltd, Tokyo, Japan).

Experimental procedure

The birds were fed once daily on 80 g of the appropriate test diet, an amount that was completely consumed on every occasion. They received each of the experimental diets for a period of 8 d and daily collections of urine and faeces were made on the last 3 d. For convenience of management within the isolators the diets were given in a systematic order: casein, casein + $NaHCO_3$, N-free, N-free + $NaHCO_3$. Since the birds were nearly mature, weight changes during the course of the experiment were expected to be small, so that the observed effects of the treatments were unlikely to be influenced by the order of their application. Body-weights were recorded daily.

Table 1. *Effect of an alkali load on daily nitrogen excretion (mg/kg body-weight) of germ-free (GF) or conventional (CV) chickens given successively a diet containing 200 g casein/kg or a N-free diet.*

(Values are means of three birds)

	N-free diet (*a*)		N-free diet + 9 mmol $NaHCO_3$/d (*b*)		Main effect of environment (GF–CV)		Main effect of diet (*b*)–(*a*)		
	GF	CV	GF	CV	Mean	SE (4 df)	Mean	SE (4 df)	Interaction†
Urinary N:									
Total N	172	120	140	103	+44NS	44	−25NS	18	−8NS
Uric Acid-N	136	88	112	73	+44NS	42	−20NS	14	−5NS
Ammonia-N	21	26	6	7	−3·1NS	3·9	−16·8*	4·0	+2·3NS
Faecal N	51	47	31	44	−4·5NS	10·3	−10·8NS	4·6	−8·5NS
Total N excreted	223	168	171	147	+39NS	51	−37NS	19	−16NS

	Casein diet (*c*)		Casein diet + 20 mmol $NaHCO_3$/d (*d*)		Main effect of environment (GF–CV)		Main effect of diet (*d*)–(*c*)		
	GF	CV	GF	CV	Mean	SE (4 df)	Mean	SE (4 df)	Interaction†
Urinary N:									
Total N	458	586	463	671	−168NS	65	+45NS	36	−40NS
Uric acid-N	354	469	354	519	−140*	48	+25NS	35	−25NS
Ammonia-N	68	90	22	34	−17·5NS	6·6	−51*	5·4 (2 df‡)	+4·6
Faecal N	145	142	115	139	−10·5NS	29·4	−17·2NS	13·5	−13·5NS
Total N excreted	603	729	577	809	−179NS	93	+27NS	30	−54NS

† Standard error of the interaction equals the standard error of the effect of diet.
‡ Degrees of freedom reduced to 2 due to evidence of variance heterogeneity.
Significance levels: NS, not significant, $P > 0{\cdot}05$; *$P \leqslant 0{\cdot}05$.

Statistical analysis

Inspection of the results showed that the excretion of nitrogenous products was greater when the diet contained casein than when the N-free diet was given. There was more variation in the results for casein so the results for the two diets were analysed separately. A split plot analysis of variance was carried out taking chickens as main plots and diet periods as sub-plots. Hence the effect of environment was assessed using the Between Chicken Variation with 4 df and the effects of $NaHCO_3$ and its interaction with environment was assessed using the Within Chicken Variation, also with 4 df. Standard errors based on these error variances are given in Table 1.

Variation in amino acid composition values of the faeces of chickens given the N-free and casein diets without extra $NaHCO_3$ (Table 2) did not show gross differences between diets, as described previously. The results were analysed in a similar way, the within chicken comparison being between protein-free and casein diets instead of between the presence and absence of $NaHCO_3$.

RESULTS

Throughout the experimental period the birds in the germ-free isolators remained uncontaminated according to the tests described by Coates *et al.* (1963).

At the beginning of the experiment the GF birds were heavier (1755, 1774 and 1856 g) than those in the conventional environment (1640, 1514 and 1700 g). During the 16 d when they received the casein diet without and with the alkali load the average daily gain of the

Table 2. *Mean values (g/160 g N) for amino acids and ammonia in faeces of three germ-free (GF) or conventional (CV) chickens given a N-free diet or one containing 200 g casein/kg*

	N-free diet (*a*)		Casein diet (*b*)		Main effect of environment (GF–CV)		Main effect of diet (*b*)–(*a*)		Interaction†
	GF	CV	GF	CV	Mean	SE (4 df)	Mean	SE (4 df)	
Lysine	6·7	19·8	28·2	20·8	−2·9NS	2·28	11·3*	2·65	10·2*
Histidine	4·6	9·7	12·1	9·4	−1·2NS	1·35	3·6*	1·08	3·9*
Ammonia	21·6	20·8	25·0	23·6	1·1NS	1·20	3·1*	1·03	0·3NS
Arginine	23·4	22·6	28·5	16·1	6·7NS	2·95	−0·7NS	3·34	5·8NS
Aspartic acid	62·2	64·6	77·2	68·4	3·2NS	1·47	9·4NS	5·14	5·6NS
Threonine	57·2	51·8	59·7	39·5	12·8**	2·66	−4·9NS	3·14	7·4NS
Serine	88·7	55·8	146·5	120·9	29·2**	3·74	61·5***	4·37	−3·6NS
Glutamic acid	77·8	86·8	235·8	201·2	12·8NS	8·79	136·2***	11·25	21·8NS
Proline	61·3	42·5	100·5	64·8	27·3**	5·12	30·7**	4·57	8·4NS
Glycine	50·5	44·2	33·7	29·9	5·0NS	2·38	−15·6**	1·92	−1·3NS
Alanine	31·6	38·6	33·4	37·6	−5·6*	1·76	0·4NS	1·76	1·4NS
Cystine	54·4	34·0	28·3	33·7	7·5**	0·88	−13·2NS	6·35	−12·9NS
Valine	50·1	48·8	49·2	51·0	−0·3NS	3·89	0·6NS	2·87	−1·6NS
Methionine	5·2	10·3	12·6	11·8	−2·1NS	1·47	4·4**	0·65	2·9*
Isoleucine	35·6	33·2	70·3	59·4	6·6*	1·90	30·4***	0·37	4·3***
Leucine	52·8	43·5	45·5	37·5	8·7**	1·58	−6·7*	2·42	−0·6NS
Tyrosine	42·8	43·2	32·4	21·4	5·3NS	5·35	−16·1**	3·45	5·7NS
Phenylalanine	44·7	41·8	22·3	17·6	3·8NS	3·10	−23·3**	3·68	0·9NS
%N recovered as amino acids	77	66	91	77	12·3**	2·32	12·7**	1·54	1·3NS

† Standard error of the interaction equals the standard error of the effect of diet.
Significance levels: NS, not significant, $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

CV birds was 2 g compared with 8 g gained by the GF group. During the corresponding period on the N-free diet, however, the average body-weight loss by the GF birds was 25 g/d compared with only 15 g/d by the CV chickens.

The results of the analyses of urine and faeces expressed in terms of body-weight, are shown in Table 1. During the period on the casein diet total N in the excreta was higher in the conventional than in the germ-free environment. Total urinary N excretion was also higher, but the differences were not quite significant statistically. The higher N retention was reflected in the greater gain in body-weight by the GF birds. Uric acid excretion paralleled total urinary N in all groups and was significantly higher in the conventional environment. In both environments urinary ammonia excretion was reduced when sodium bicarbonate was included in the diet, but none of the other nitrogenous urinary constituents was affected. During the period on the N-free diet the total N excretion was higher, though not significantly, in the germ-free state, and the body-weight loss was greater in the GF than in CV birds. There was again a marked drop in urinary ammonia excretion when the alkali load was imposed ($P < 0.05$ for the two environments combined).

Amino acids were measured in faecal hydrolysates, thus the values given include those derived from protein and peptides as well as the free amino acids in the original material. During the periods when no alkali was given, the total amounts of amino acids (mg/bird per d) in the faeces of GF and CV birds respectively were 463 and 332 on the N-free diet and 1707 and 1228 on the casein diet. The difference between diets was significant ($P < 0.05$) but that between environments was not ($P < 0.1$). The amino acid compositions (g/160 g N) are given in Table 2. In general the values on the casein diet were similar to or higher than

those on the N-free diet; the converse was true of glycine, leucine, tryosine and phenylalanine.

The results for cystine were unusual. A significant over-all effect of environment was indicated despite the fact that with the protein-free diet more cystine was excreted by the GF than by the CV chicks, whereas with the casein diet CV chicks excreted more. This over-all result appeared to be due to the chance occurrence of very little variation 'between chickens' for this amino acid.

The faeces of the GF birds on the N-free diet contained greater proportions (g/160 g N) of serine, proline, cystine, and leucine, and smaller proportions of lysine, histidine, alanine and methionine. On the casein diet the values for faeces of CV birds were almost all lower than those of the GF birds with the exception of alanine and cystine.

DISCUSSION

When the birds were given the casein diet growth was better in the germ-free environment. This is in line with general experience in this laboratory and elsewhere that GF chickens grow faster than their CV counterparts. It can be inferred that digestibility and N retention were both lower in the conventional than in the germ-free environment since, although the daily food intake (80 g) was the same for all, the CV birds excreted more N. In contrast, on the N-free diet the GF birds lost more weight and excreted more N than their conventional counterparts. Although the increase in N excretion by the GF birds was not statistically significant on this occasion it accords with earlier findings by Salter *et al.* (1974). On both diets it was evident that the difference in N excretion was almost entirely accounted for by the difference in urinary N loss between GF and CV birds.

From analysis of faeces collected during the periods with no alkali in the diet, it appears that amino acids constituted more of the N in the hydrolysate of germ-free faeces than in the corresponding conventional sample. On a N-free diet these amino acids would originate from endogenous secretions such as mucoproteins and digestive enzymes which, as Salter & Fulford (1974) pointed out, are relatively rich in threonine, serine and proline. The proportions (g/160 g N) of serine and proline were markedly reduced and that of threonine somewhat lower in the conventional faeces. Thus these findings support the hypothesis that when N intake is low the gut micro-organisms provide a means of conserving endogenous N, probably by release and subsequent absorption of ammonia from amino acids in the lower gut. There was evidence of synthesis of lysine, histidine, alanine and methionine which, if absorbed, could have been of benefit to the host. Faeces collected when the birds were given the casein diet contained significantly greater amounts of amino acids than those during the period on the N-free diet, indicating that the dietary protein had not been completely digested or that the digestion products had not been fully absorbed. The proportions of most amino acids were reduced in the presence of the gut microflora, probably also through reactions resulting in deamination. However, any N-sparing effect of the micro-organisms would be of doubtful nutritional importance since the dietary intake of protein was obviously adequate.

Urinary ammonia excretion fell sharply in both environments when $NaHCO_3$ was included in either of the diets. Okumura & Tasaki (1968) showed that 20 mmol $NaHCO_3$ was more than adequate to depress urinary ammonia excretion associated with acid-base balance of chickens eating 80 g of the diet with 200 g casein/kg. Thus the fall from 68 to 22 mg urinary ammonia/kg body-weight in the GF birds represents the regulatory effect on the acid-base balance when there is no interference from microbial action. Similarly, the fall from 90 to 34 mg urinary ammonia/kg body-weight in the CV birds accounts for the combined effects of alkali load and gut microflora. In this instance the ammonia excretion

was slightly higher, and the difference without and with alkali marginally greater, than were found with GF birds but these effects were not significant. In the birds given the N-free diet no difference was found between urinary ammonia excretion in the two environments and the fall in response to the alkali load was virtually the same in both. Uric acid and ammonia did not account for all the urinary N, the other major nitrogenous components presumably being urea and creatinine. In all instances the amount of N not in the form of ammonia or uric acid was increased by supplementation with $NaHCO_3$, especially on the casein diet. A possible explanation may be that when an alkali load is imposed some of the ammonia that would have been excreted as such is converted to the more neutral urea. It is clear, however, from these findings that the level of dietary protein and the ingestion of alkali have much greater effects than the activities of the microflora on urinary ammonia excretion.

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