Br. J. Nutr. (1973), **29**, 423

A comparison of fractions prepared from navy (haricot) beans (*Phaseolus vulgaris* L.) in diets for germ-free and conventional chicks

By D. HEWITT and MARIE E. COATES

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT

and M. L. KAKADE* and I. E. LIENER

Department of Biochemistry, College of Biological Sciences, University of Minnesota, St Paul, Minnesota 55101, USA

(*Received* 19 *June* 1972 – *Accepted* 22 *September* 1972)

1. Germ-free and conventional chicks were given diets containing raw or heated navy (haricot) (*Phaseolus vulgaris* L.) bean meal, or the heated meal supplemented with one of two fractions from raw navy beans: F3 was a preparation of navy bean trypsin inhibitor; F4 was a toxic factor known to depress the growth of rats. After 2 weeks, body-weights and pancreas weights were determined and proteolytic and amylolytic enzymes in the pancreas were assayed. The contents of the small intestine were collected and analysed for nitrogenous compounds.

2. In germ-free chicks final body-weights were not affected by F3 and only slightly depressed by F4. A significant depression of 12% occurred when raw meal was given. In conventional chicks depressions in final body-weights of 13·5, 26·5 and 48% were recorded with F3, F4, and raw meal respectively.

3. In birds given either raw meal or F3, there was an increase in size of the pancreas and in its concentration of trypsin, but a reduction in concentrations of chymotrypsin and amylase. The concentration of nitrogen in the insoluble fraction of the intestinal contents was increased by raw meal and F3 in both environments.

4. It was concluded that the presence of the gut microflora aggravated the growth-depressing effect on chicks of raw navy-bean meal. In the absence of the gut flora neither factor had any effect on growth. Possible explanations are discussed.

Many legumes in the raw state support lower growth rates than the respective heated meals. As well as growing slowly, animals given raw legumes often have large pancreases. Miller & Coates (1966) and Coates, Hewitt & Golob (1970) reported that the growth depression caused by raw soya-bean meal in germ-free chicks was significantly less than that in conventional chicks. Pancreatic enlargement occurred in both environments to the same extent. A preliminary communication (Hewitt & Coates, 1969) showed that this was also true for raw navy (haricot) bean (*Phaseolus vulgaris* L.). The effects of raw navy bean may be caused by protease inhibitor(s) and haemagglutinin(s), although amylase inhibitors (Liener, 1969), small amounts of cyanogenetic glucosides (Montgomery, 1969) or other toxic substances present in the beans may be involved. Kakade & Evans (1965*a*), using the method of Honavar, Shih & Liener (1962), separated a saline extract of raw navy beans into five fractions. The work reported here is a detailed study on germ-free and conventional chicks of the effects of raw and heated navy-bean meals and of two of these fractions (F3 and F4). F3 was rich in trypsin inhibitor and F4 was the most active in depressing growth in rats.

* Present address: Land O'Lakes Inc., Minneapolis, Minnesota 55413, USA.

MATERIALS AND METHODS

Chicks and experimental treatments

Germ-free chicks were produced and reared as described by Coates *et al.* (1970), using eggs from the National Institute for Research in Dairying flock of Light Sussex hens and Rhode Island Red cocks. Briefly, towards the end of incubation the outsides of the eggs were disinfected with peracetic acid. Incubation of half the eggs was continued in the stainless-steel isolators where the germ-free chicks were reared. The rest were returned to the incubator to provide conventional controls, which were reared in a specially designed room where environmental conditions of temperature and humidity, similar to those in the isolators, were maintained. Within the conventional environment each experimental diet, sterilized by γ-radiation at 5 Mrad, was given *ad lib.* to duplicate groups of 1-d-old chicks, five per group. Each group was reared in a stainless-steel cage. The cages were arranged in two blocks of four. Two similar blocks were set up in the germ-free environment. The chicks were weighed weekly. When the chicks were 14 or 15 d old, six birds from each dietary treatment were anaesthetized with diethyl ether and the contents of the small intestine were collected. Finally, all the chicks were killed with diethyl ether and the pancreases were removed, weighed and stored at $-10°$ until required for analysis.

Trypsin, chymotrypsin and amylase were assayed, and nitrogen was determined in each pancreas. The contents of the small intestine were separated into soluble and insoluble fractions before determination of their nitrogenous constituents.

During the experiment swabs were taken from birds in the isolators to check for contamination by micro-organisms.

Diets

The composition of the diets is given in Table 1. Raw and heated navy-bean meals were used. The raw beans (Sanilac variety; Clare & Glen Harrington, Akron, Michigan, USA) were ground to a fine meal. The heated sample was prepared by autoclaving (at 121° and 1·06 kg/cm^2 for 5 min) the raw meal, which was spread on shallow steel trays. These were the optimum conditions found by Kakade & Evans (1965*b*) for improving the nutritive value of raw navy-bean meal. The diets contained 50% raw or heated navy-bean meal, or 50% heated meal and either trypsin inhibitor (F3), 8 g/kg, or 'toxic factor' (F4), 15 g/kg. These minor supplements were added at the expense of starch. The levels of protein and sulphur amino acids in the diets were similar to those in the diets described by Coates *et al.* (1970). It was, however, considered that the use of casein in the diets might introduce an amino acid imbalance due to its high content of lysine relative to arginine. To counteract this, arginine was included in the diet to raise its level relative to lysine to that suggested by D'Mello & Lewis (1970). This precaution should allow comparison of the present results with those of Coates *et al.* (1970) with soya-bean meal.

Navy bean fractions F3 and F4, corresponding to a trypsin inhibitor-enriched fraction and a toxic fraction devoid of anti-tryptic activity respectively, were prepared essentially by the method previously described by Kakade & Evans (1965*a*). In the

Table 1. *Composition of the diet (per kg)*

Casein	100 g
Salt mixture*	60 g
Glycine	5 g
L-methionine	2·9 g
L-arginine hydrochloride	4·84 g
myo-Inositol	1 g
Choline chloride	1·5 g
B-vitamin triturate in glucose†	8 g
Maize oil	41·6 g
Cholecalciferol in maize oil (0·4 mg/g)	0·4 g
α-Tocopheryl acetate in maize oil (10 mg/g)	4 g
Menaphthone in maize oil (5 mg/g)	4 g
Rovimix A (Roche Products Ltd, Welwyn Garden City) containing 97·5 μg retinol/mg	0·209 g
Cyanocobalamin solution (100 μg/ml)	0·8 ml
Navy-bean meal	500 g
Fractions	Variable (see p. 424)
Maize starch	to 1 kg

* Contained (parts by weight): $CaCO_3$ 2566, $CaHPO_4.2H_2O$ 2566, KH_2PO_4 2000, NaCl 1300, $MgSO_4.H_2O$ 400, $FeSO_4.7H_2O$ 100, $MnSO_4.4H_2O$ 40, $ZnSO_4.7H_2O$ 20, $CuSO_4.5H_2O$ 2·4, KI 5·6.

† To provide (mg/kg diet): biotin 0·8, pteroylmonoglutamic acid 6, thiamin hydrochloride 12, pyridoxine hydrochloride 16, riboflavin 24, calcium pantothenate 60, nicotinic acid 160.

Table 2. *Trypsin inhibitor activity of the diets*

(F3 was the trypsin inhibitor fraction and F4 was the toxic fraction, see p. 424)

Diet	Inhibitor activity (units*/mg)
Raw meal	3·4
Heated meal	0·1
Heated meal + 8 g F3/kg	3·3
Heated meal + 15 g F4/kg	0·0

* One unit of inhibitor activity is the amount that produces a reduction in extinction at 410 nm, equivalent to the production of 0·01 μmol *p*-nitroaniline by trypsin.

latter procedure the preparation of F3 involves treatment of an acid (pH 4) extract of the beans with a mixture of bentonite and Celite to absorb the trypsin inhibitor. The latter is eluted with pyridine solution (100 ml/l), dialysed and, in the modified procedure used here, subsequently heated at 80° for 5 min to precipitate inactive protein. The supernatant solution was freeze-dried and constitutes the material used in these studies. The amount of F3 added to the diet was equivalent to the amount contributed by 50% raw meal, as shown in Table 2 which gives the trypsin inhibitor activity of the diets. Trypsin inhibitor activity was determined by the method of Sambeth, Nesheim & Serafin (1967) using *N*-benzoyl-DL-arginine-*p*-nitroanilide as the substrate for trypsin. One unit of inhibitor activity is the amount that produces a reduction in extinction at 410 nm, equivalent to the production of 0·01 μmol *p*-nitroaniline by trypsin. Since the mechanism of action of F4 and the identity of its active constituent(s) are unknown, no analytical procedure was available to confirm that the amount included was appropriate. The level chosen was based on the yield obtained from the raw meal.

The diets were granulated. To counteract possible losses of vitamins during sterilization by irradiation the customary supplement was quadrupled.

Collection of contents from the small intestine

Chicks were anaesthetized with diethyl ether and the contents of their small intestines were collected, as described by Coates *et al.* (1970). A glass cannula was inserted into the proximal end of the jejunum below a ligature at the point of entry of the bile duct. A second cannula was introduced into the distal ileum above a ligature at the ileo-caecal junction. Normal saline solution, 8·5 g NaCl/l, at 38° was introduced through the proximal cannula to flush out the contents through the distal cannula into a plastic bottle, cooled in ice. About 70 ml saline were used for each chick.

Analysis of gut contents

The contents from the small intestine were centrifuged at 17 500 *g* for 15 min at 5°. The supernatant fraction was decanted. The residue was resuspended in 10 ml distilled water and recentrifuged for 15 min. The supernatant fractions were combined and diluted to 100 ml with physiological saline. The residue and duplicate 10 ml samples of the supernatant fractions were dried in aluminium dishes at 105° for 18 h to determine insoluble and soluble dry matter. Equal volumes of the supernatant fractions from pairs of birds were pooled and freeze-dried to give three pooled samples per treatment. The N content of the insoluble fraction and the N, 'protein', 'peptide' and 'amino acid' contents of the pooled soluble fractions were determined as described by Coates *et al.* (1970), with the exception that the amount of soluble intestine contents fractionated on Sephadex gel filtration medium to determine 'proteins', 'peptides' and 'amino acids' was increased to contain 5 mg N. The fractions collected which corresponded to the elution of 'proteins', 'peptides' and 'amino acids' for the three samples from each treatment were pooled and taken to dryness in a rotary evaporator under reduced pressure at 45°. The residue was dissolved in 25 ml distilled water and used for the determination of N and amino acids. For amino acid analysis, a 5 ml sample was taken to dryness by rotary evaporation and then hydrolysed with 25 ml 'constant boiling' hydrochloric acid at 110° under reflux for 18 h. The hydrolysate was filtered, taken to dryness in a rotary evaporator and dissolved in the appropriate volume of pH 2·2 buffer (Moore & Stein, 1954) to give a concentration of 50 μgN/ml. The samples were stored frozen until analysed with an automatic amino acid analyser (Model JLC-5AH; JEOLCO Ltd, Tokyo, Japan). Sulphur amino acids were determined as cysteic acid and methionine sulphone in separate hydrolysates prepared from the samples after oxidation by the method of Moore (1963).

Determination of enzymes and N in the pancreas

Preliminary treatment of the pancreas. Each pancreas was homogenized in 0·15 M-NaCl, containing 1 g Triton X-100/l, and diluted with the saline to 10 ml. A 1 ml sample was taken for N determination. To another 4 ml sample, 1 ml buffer (0·2 M-tris (hydroxymethyl) methylamine, 0·25 M-$CaCl_2.2H_2O$, pH 7·95) was added and the mixture centrifuged at 4200 *g* for 20 min at 5°. The supernatant fraction was used for the assay of proteolytic enzymes. The remainder of the homogenate was also centrifuged at 4200 *g* and the supernatant fraction retained for amylase assay.

α-Chymotrypsin and trypsin determination. The supernatant fraction prepared as described above was incubated with an equal volume of enterokinase solution (500 mg/l) for 1 h at 37°. Enterokinase was prepared from pig duodenal contents as described by Kunitz (1938–9). Enzyme activities were determined according to the method of Hummel (1959) using benzoyl-L-tyrosine ethyl ester and *p*-toluene sulphonyl-L-arginine methyl ester as substrates for chymotrypsin and trypsin respectively. The change in the extinction of the substrate solution following addition of the enzyme preparation was measured with a recording spectrophotometer (Optica model CF4DR: Baird and Tatlock (London) Ltd). The unit of enzyme activity was defined as a change in extinction of 1·0/min.

α-Amylase determination. α-Amylase activity was determined by the method of Howard & Yudkin (1963). It was expressed in terms of the amount of starch hydrolysed per h.

Statistical analysis

The results, excluding those for amino acid analyses, were subjected to analysis of variance. When results for body-weight, pancreas weight, pancreas N and pancreas enzyme activities were considered, statistical analysis showed that block differences within each environment were non-significant and that variation between duplicate cages allocated to the same diet and within the same environment was not significantly greater than the variation between chicks from the same cage. Consequently for each measurement a single pooled error mean square (between chicks on the same diet and in the same environment, with 72 df) was adopted.

Single pooled error mean squares were also adopted for the results of analyses of the intestinal contents: between chicks on the same diet and in the same environment, with 40 df, for soluble and insoluble dry matter, and between pairs of chicks on the same diet and in the same environment, with 16 df, for soluble N, 'proteins', 'peptides' and 'amino acids'.

RESULTS

Chick growth and pancreas weight

Mean body-weights and pancreas weights are presented in Table 3. In the isolators, which remained germ-free throughout the experiment, chicks given the raw meal were lighter than those given heated meal ($P < 0{\cdot}01$). Inclusion of F3 and F4 with heated meal had no significant effect on chick weight. In conventional chicks, comparison with the heated meal diet showed that the raw meal depressed growth ($P < 0{\cdot}001$) much more than it did in germ-free chicks. There was also a reduction in body-weight when F3 was given to conventional chicks ($P < 0{\cdot}002$) and a greater reduction when F4 was given ($P < 0{\cdot}001$).

The weights of the pancreases of germ-free chicks given heated meal or F4 were not significantly different, whereas giving raw meal or F3 resulted in larger pancreases ($P < 0{\cdot}001$). In the conventional environment, the birds given F3 had larger pancreases ($P < 0{\cdot}001$) and those given raw meal or F4 had smaller pancreases ($P < 0{\cdot}001$ and $P < 0{\cdot}01$ respectively) than birds given heated meal. When pancreas weight was

Table 3. *Effects of raw and heated navy-bean meal and navy-bean fractions on mean body-weight at 13 d old and pancreas weight at 14–15 d old of germ-free and conventional chicks*

(Each value is the mean for two groups of five chicks. F3 was the trypsin inhibitor fraction and F4 was the toxic fraction, see p. 424)

	Germ-free chicks			Conventional chicks		
		Pancreas wt			Pancreas wt	
Diet	Body-wt (g)	g	g/kg body-wt	Body-wt (g)	g	g/kg body-wt
Raw meal	116	0·75	6·2	65	0·47	6·9
Heated meal	132	0·52	3·7	125	0·64	4·6
Heated meal + 8 g F3/kg	133	0·86	6·1	108	0·90	7·5
Heated meal + 15 g F4/kg	125	0·45	3·5	92	0·51	5·0

	Body-wt (g)	Pancreas wt	
		g	g/kg body-wt
SE of a mean (with 72 df)	3·6	0·033	0·27
Least significant difference ($P = 0·05$)	10·1	0·092	0·77

expressed as a proportion of the body-weight the values for chicks given raw meal or F3 were roughly equal and significantly greater than for chicks given heated meal or F4. The extent of pancreatic enlargement was similar in both environments although the values for germ-free chicks were generally lower than those for conventional chicks ($P < 0·001$).

Analysis of gut contents

The amounts of dry matter and N in the soluble and insoluble fractions of the small intestine contents are given in Table 4. In general there was more insoluble material ($P < 0·001$) and more insoluble N in conventional than in germ-free samples. Compared with birds given heated meal, the concentration of N in the insoluble fraction from chicks given raw meal or F3 was significantly higher ($P < 0·05$) in both environments. The N concentration in the insoluble fraction was not affected by F4. There was generally more soluble material ($P < 0·01$) and soluble N ($P < 0·05$) in gut contents from conventional than from germ-free chicks. Between diets the differences were small, except in conventional chicks where F4 reduced the amount of soluble material to that in germ-free birds. The effect of diet on the concentration of N in the soluble contents was not consistent.

The amounts of the three fractions, 'protein', 'peptide' and 'amino acid', in the soluble N of the small intestine contents are also given in Table 4. The most noticeable treatment effect was in conventional chicks given F4, where the 'amino acid' fraction was increased and the 'protein' fraction decreased ($P < 0·001$ for each) in comparison with fractions from chicks given heated meal. The concentrations of five representative amino acids in the fractions are given in Table 5. Differences between treatments were inconsistent, but there were marked differences between the fractions. There was less threonine in the 'amino acid' than in the 'peptide' fractions, less lysine in the 'protein' and more glutamic acid in the 'peptide' than in the other fractions.

Table 4. *Dry weight and nitrogen in insoluble and soluble fractions and 'proteins', 'peptides' and 'amino acids' in soluble fraction of small intestine contents of germ-free and conventional chicks reared to 2 weeks on diets containing navy-bean meal and navy-bean fractions*

(Mean values with their standard errors for six chicks, except for soluble N, 'proteins', 'peptides' and 'amino acids' which are means for three pooled samples each from two chicks. For insoluble N a separate SE is given for each diet because of heterogeneity in the error variances. F_3 was the trypsin inhibitor fraction and F_4 was the toxic fraction, see p. 424)

		Insoluble contents					Soluble contents					
			N					N				
Type of chick	Diet	Dry wt (g/kg body-wt)	mg/kg body-wt Mean	mg/kg body-wt SE	g/kg dry matter Mean	g/kg dry matter SE	Dry wt (g/kg body-wt)	mg/kg body-wt	g/kg dry matter	'Proteins', 'Peptides', 'Amino acids' (g leucine equivalent/g N)		
Germ-free	Raw meal	2·69	26·1	3·4	9·8	0·6	1·88	84·7	46·1	4·39	0·82	1·06
	Heated meal	2·88	15·0	0·5	5·3	0·3	1·81	66·3	36·2	4·00	1·51	1·61
	Heated meal + 8 g F_3/kg	2·62	21·1	2·5	8·3	0·6	2·01	87·0	43·5	4·03	1·04	1·68
	Heated meal + 15g F_4/kg	3·09	19·6	3·4	6·7	0·6	2·02	90·3	43·0	3·55	1·05	1·30
Conventional	Raw meal	4·79	74·8	12·6	15·1	1·2	2·43	122·9	49·9	4·36	1·10	1·74
	Heated meal	4·74	34·1	8·1	7·2	0·8	2·69	99·5	37·4	4·02	0·91	1·88
	Heated meal + 8g F_3/kg	3·46	35·8	4·9	10·3	1·0	2·52	106·5	42·0	3·78	0·94	1·99
	Heated meal + 15g F_4/kg	3·62	27·5	3·4	7·6	0·3	2·05	90·1	44·2	3·02	1·57	2·79
SE of a mean		0·47 (40 df)					0·22 (40 df)	13·1 (16 df)	3·3 (16 df)	0·18 (16 df)	0·11 (16 df)	0·17 (16 df)
Least significant difference ($P = 0·05$)		1·35					0·63	39·2	9·9	0·54	0·34	0·51

Table 5. *Concentrations of selected amino acids (μmol/mg nitrogen) in the 'proteins', 'peptides' and 'amino acids' fractions from the soluble contents of the small intestine of germ-free and conventional chicks reared to 2 weeks on diets containing navy-bean meal and navy-bean fractions*

(Values are for pooled samples from six chicks. F3 was the trypsin inhibitor fraction and F4 was the toxic fraction, see p. 424)

	Germ-free				Conventional			
Amino acid	Raw	Heated	Heated +F3	Heated +F4	Raw	Heated	Heated +F3	Heated +F4
				'Proteins'				
Threonine	3·32	2·99	3·48	3·27	3·13	3·22	3·14	3·52
Glutamic acid	5·82	7·01	6·90	6·94	6·33	7·37	6·14	6·85
Lysine	2·19	1·90	2·21	2·16	2·38	2·16	2·04	2·16
Cystine	0·32	0·27	0·52	0·09	0·27	0·17	0·19	0·25
Methionine	0·19	0·29	0·31	0·13	0·28	0·20	0·15	0·32
				'Peptides'				
Threonine	3·62	3·56	3·89	3·79	3·83	4·09	3·42	4·02
Glutamic acid	10·21	9·76	9·69	10·02	11·88	11·49	9·02	9·65
Lysine	3·70	3·17	3·75	3·66	4·11	3·76	2·46	2·66
Cystine	0·42	0·40	1·02	0·71	0·50	0·88	0·23	0·25
Methionine	0·44	0·30	0·55	0·46	0·51	0·57	0·32	0·20
				'Amino acids'				
Threonine	2·71	2·47	2·91	2·48	2·65	3·19	2·57	3·22
Glutamic acid	6·41	5·75	3·24	4·83	7·24	5·65	6·13	8·56
Lysine	2·53	2·45	4·55	2·11	3·27	3·10	2·62	3·34
Cystine	0·30	0·26	0·39	0·35	0·29	0·40	0·26	0·27
Methionine	0·47	0·56	0·76	0·72	0·42	0·42	0·33	0·30

Studies on the pancreas

N concentrations in the pancreas are given in Table 6. In conventional chicks there was less pancreatic N than in germ-free chicks when the diet contained raw meal ($P < 0{\cdot}001$), F3 or F4, but the same amount of N when the diet contained heated meal. Analysis of variance showed that the main effect of environment was significant ($P < 0{\cdot}001$), as was the interaction between diet and environment ($P < 0{\cdot}05$). The only significant difference between dietary treatments occurred in the conventional environment, where the values were lower with raw meal than with heated meal or F3.

The activities of trypsin, chymotrypsin and amylase in the pancreas are also given in Table 6. For all diets the concentration of each enzyme was higher in germ-free than in conventional chicks but the effect of environment was significant ($P < 0{\cdot}05$) only for trypsin, when raw meal was given, and for α-amylase when heated meal was given. Compared with heated meal the effects of raw meal and F3 were as follows. Trypsin activity was increased in germ-free chicks given raw meal ($P < 0{\cdot}002$) and in germ-free and conventional chicks given F3 ($P < 0{\cdot}001$ and $< 0{\cdot}05$ respectively). α-Chymotrypsin activity was decreased in germ-free birds given raw meal ($P < 0{\cdot}001$) or F3 ($P < 0{\cdot}01$). Amylase activity was also decreased by raw meal and by F3 ($P < 0{\cdot}05$) in germ-free and conventional chicks. F4 was without effect on

Table 6. *Nitrogen, trypsin, chymotrypsin and amylase in pancreases of germ-free and conventional chicks reared to 2 weeks on diets containing navy-bean meal and navy-bean fractions*

(Mean values with their standard errors for two groups of five chicks.
F_3 was the trypsin inhibitor fraction and F_4 was the toxic fraction, see p. 424)

Type of chick	Diet	N (mg/g fresh wt)	Activity/g pancreas: Trypsin (units*)	Activity/g pancreas: α-Chymotrypsin (units*)	Activity/g pancreas: α-Amylase (g starch hydrolysed/h)	Total activity/pancreas: Trypsin (units*)	Total activity/pancreas: α-Chymotrypsin (units*)	Total activity/pancreas: α-Amylase (g starch hydrolysed/h)
Germ-free	Raw meal	29·2	50·1	47·9	236	38·0	36·3	176
	Heated meal	26·6	28·5	90·6	354	14·8	47·8	186
	Heated meal + 8 g F_3/kg	29·1	53·6	57·2	252	46·9	49·4	218
	Heated meal + 15 g F_4/kg	28·1	30·9	100·4	316	13·2	45·4	144
Conventional	Raw meal	23·5	35·6	46·7	192	19·0	26·8	94
	Heated meal	26·6	26·3	74·9	260	16·8	47·7	164
	Heated meal + 8 g F_3/kg	26·8	41·4	44·9	196	36·9	40·3	178
	Heated meal + 15 g F_4/kg	25·2	23·4	73·9	294	12·4	37·2	148
SE of a mean (with 72 df)		1·0	4·6	8·6	21	3·6	6·0	17
Least significant difference ($P = 0·05$)		2·8	13·0	24·3	59	10·2	16·9	48

* A unit gives a change in extinction of 1·0/min.

trypsin and chymotrypsin activities; it increased amylase activity in conventional chicks and had the opposite effect in germ-free chicks. When the total quantities of chymotrypsin and amylase in the pancreas were considered, the effect of diet decreased because those chicks with lower enzyme concentrations had larger pancreases (Table 3). In both environments supplementation of heated meal with F3 or F4 did not have any significant effect on total chymotrypsin activity. In germ-free chicks F3 increased and F4 decreased total amylase activity. On the raw meal diet, reductions in total amounts of chymotrypsin ($P < 0.02$) and amylase ($P < 0.001$) occurred in conventional chicks. In contrast, the total activities of trypsin in germ-free chicks given raw meal, and in both types of chick given F3, were higher ($P < 0.001$) than in corresponding birds given heated meal.

DISCUSSION

Many legumes have been shown to support poor growth or result in weight loss unless they have been adequately heated. In the experiment reported here the growth depression caused by raw navy-bean meal was much less in the germ-free than in the conventional environment. This extends the observations of Miller & Coates (1966) and Coates *et al.* (1970), who reported similar results with soya-bean meal, and supports their suggestion that the gut microflora is concerned in the severe growth depression caused by the raw meals. To account for the comparatively mild effect in germ-free chicks Coates *et al.* (1970) suggested that a material present in an innocuous form in the raw meal may be converted into a toxin by microbial action in the gut. A second postulate (Jayne-Williams & Coates, 1969) is that impaired protein digestion due to the trypsin inhibitor could result in an accumulation of undigested protein in the lower gut, encouraging the establishment of micro-organisms involved in the growth depression. To test these hypotheses two fractions were selected from five prepared by Kakade & Evans (1965*a*) from raw navy beans. F3 was a preparation of the navy-bean trypsin inhibitor. F4 caused severe growth depression in tests with rats and, although it had some haemagglutinating activity, Kakade & Evans (1965*a*) found that its toxicity was not correlated with this.

In our experiment raw navy-bean meal caused a slight depression of growth in germ-free chicks but the responsible factor was not present in F3 or F4, neither of which affected growth of germ-free chicks. Both fractions reduced the body-weights of conventional chicks, F4 to a greater extent than F3. Thus both theories proposed to account for the effect of environment on the toxicity of raw legumes are supported by these results. The large growth depression recorded when F4 was given to conventional chicks could have been caused by a constituent produced by the action of the gut microflora on a non-toxic precursor. It is also possible that F4 stimulated a particular harmful organism(s) present in the gut. The smaller growth depression caused by F3 indicates that trypsin inhibition is also concerned in the general effect of raw navy beans on growth of conventional chicks. The results given in Table 4 suggest that inhibition of proteolysis was less than that described by Coates *et al.* (1970) with raw soya-bean meal, because the proportion of protein in the soluble fraction of the

gut contents was not greatly increased in birds given raw meal or F3. However, there was more N in the insoluble contents from birds given raw compared with heated navy-bean meal and, to a lesser extent, in those from chicks given F3.

In the soluble fractions no consistent effect of the dietary treatments on amino acid concentrations was apparent. If these results can be extended to the excreta, they do not indicate any effect of diet on amino acid excretion. In contrast, in an experiment restricted to 2 d to minimize adaptation of the pancreas to the trypsin inhibitor, Kakade, Arnold, Liener & Waibel (1969) found that conventional chicks given F3 excreted large amounts of protein-bound amino acids in their droppings compared with chicks given heated inhibitor. In particular, when raw inhibitor was given, 55·5 % of the cystine intake was excreted compared with only 23·7 % when heated inhibitor was given. In our experiment, which lasted 2 weeks, there was no clear indication of more cystine in the soluble material of the small intestinal contents of birds given F3 or raw meal. It is possible, however, that there may have been more in the insoluble fraction of intestinal contents, or that the high cystine excretion reported by Kakade *et al.* (1969) was of urinary origin.

In most treatment groups the concentration of N in the pancreas was fairly similar (Table 6), but conventional chicks given raw meal showed the lowest values. This low N content may have been associated with delayed development of these birds since Kakade, Barton, Schaible & Evans (1967) found in chicks from 1–8 d old that the amount of N in the pancreas increased with age.

The influence of the type of navy-bean meal on pancreatic trypsin and amylase activities (Table 6) was similar to that reported by previous workers (Kakade *et al.* 1967; Coates *et al.* 1970) for soya-bean meals. When the diet contained raw instead of heated meal the activity of trypsin per unit weight was increased and that of amylase was decreased. In the present experiment pancreatic chymotrypsin concentration, like amylase concentration, was lower when the diet contained raw rather than heated meal. Chicks given heated meal with F3 exhibited enzyme activities similar to those of chicks given raw meal. Thus, although the purified trypsin inhibitor did not affect the amount of insoluble N in the gut contents as much as raw meal, its effect on pancreatic enzyme activities was as great as that of raw meal. This could occur if, compared with the raw meal, the purified inhibitor passed more rapidly along the intestine and, although eliciting a similar pancreatic response, it may have had a less severe effect on digestion owing to more rapid inactivation. Alternatively, the diet containing raw meal may have contained more trypsin inhibitor than the diet supplemented with F3, despite the results in Table 2, if 'bound' inhibitors are present in raw meal as was suggested for the soya-bean by Kakade, Simons, Liener & Lambert (1972). It appears that the effect of raw navy-bean meal on pancreatic enzyme activities is due solely to the trypsin inhibitor it contains since supplementation of the heated meal with F4 had no effect on pancreatic enzyme activities. Coates *et al.* (1970) reported that pancreatic trypsin activity of germ-free birds given raw soya-bean meal was less than that of their conventional counterparts. This was not so with raw navy-bean meals and, in our further experiments with soya-bean meals, environment has been without effect on pancreatic trypsin activity.

It is often assumed that trypsin inhibitor(s) or haemagglutinin(s) are responsible for the harmful effects of raw legumes, since both substances are inactivated by heat. Under the conditions of our experiment growth depression was not due to a direct action on the chick of trypsin inhibitor, since growth of germ-free birds was unaltered by F3. Instead, its growth-depressing effect appears to have been mediated through the gut microflora, because the conventional controls grew significantly less well. The same conclusion may be drawn for F4. Our findings also support the observation of Kakade & Evans (1965*a*) that the major growth-depressing agent in navy beans was concentrated in F4. Its mechanism of action remains a matter for conjecture. Jayne-Williams & Hewitt (1972), who found raw navy-bean meal lethal to conventional but not to germ-free quail, suggested that haemagglutinin(s) might have altered the intestinal mucosa and allowed bacteria to invade the tissues. As Kakade & Evans (1965*a*) found in rats that the haemagglutinin activity of their fractions was unrelated to the effect on growth, it seems likely that further explanation must be sought to account fully for the poor performance of conventional chicks given F4. Our observation that in conventional birds the 'amino acids' in the soluble fraction of the intestinal contents were increased at the expense of 'protein' might indicate that F4 had altered the course of digestion in the presence of the microflora. However, the relevance of this observation is doubtful since the effect was not seen in conventional chicks given raw meal.

It is clear that, under the conditions of this experiment, much of the growth depression caused in conventional chicks by raw navy-bean meal was dependent on interactions between the gut microflora and the protease inhibitor(s) and other component(s) of the navy beans. The mechanisms of these interactions remain to be elucidated.

We are grateful to Dr D. J. Jayne-Williams for sterility checks on the germ-free birds, Mr D. R. Westgarth for advice on the statistical analyses, and Mrs C. A. Ewart for help with the analyses. The production and care of the chicks was supervised by Mr J. P. Fordham. That portion of this research which was performed at the University of Minnesota was supported by grant No. AM 13869 from the National Institute of Arthritis and Metabolic Diseases, US Public Health Service.

REFERENCES

Coates, M. E., Hewitt, D. & Golob, P. (1970). *Br. J. Nutr.* **24**, 213.
D'Mello, J. P. F. & Lewis, D. (1970). *Br. Poult. Sci.* **11**, 367.
Hewitt, D. & Coates, M. E. (1969). *Proc. Nutr. Soc.* **28**, 47A.
Honavar, P. M., Shih, C.-V. & Liener, I. E. (1962). *J. Nutr.* **77**, 109.
Howard, F. & Yudkin, J. (1963). *Br. J. Nutr.* **17**, 281.
Hummel, B. C. W. (1959). *Can. J. Biochem.* **37**, 1393.
Jayne-Williams, D. J. & Coates, M. E. (1969). In *International Encyclopaedia of Food and Nutrition*, Vol. 17, part 1, p. 241 [D. P. Cuthbertson, editor]. Oxford: Pergamon Press.
Jayne-Williams, D. J. & Hewitt, D. (1972). *J. appl. Bact.* **35**, 331.
Kakade, M. L., Arnold, R. L., Liener, I. E. & Waibel, P. E. (1969). *J. Nutr.* **99**, 34.
Kakade, M. L., Barton, T. L., Schaible, P. J. & Evans, R. J. (1967). *Poult. Sci.* **46**, 1578.
Kakade, M. L. & Evans, R. J. (1965*a*). *J. agric. Fd Chem.* **13**, 450.
Kakade, M. L. & Evans, R. J. (1965*b*). *Br. J. Nutr.* **19**, 269.

Kakade, M. L., Simons, N. R., Liener, I. E. & Lambert, J. W. (1972). *J. agric. Fd Chem.* **20**, 87.
Kunitz, M. (1938–9). *J. gen. Physiol.* **22**, 447.
Liener, I. E. (1969). In *Toxic Constituents of Plant Foodstuffs*, p. 430 [I. E. Liener, editor]. New York and London: Academic Press.
Miller, W. S. & Coates, M. E. (1966). *Proc. Nutr. Soc.* **25**, iv.
Montgomery, R. D. (1969). In *Toxic Constituents of Plant Foodstuffs*, p. 145 [I. E. Liener, editor]. New York and London: Academic Press.
Moore, S. (1963). *J. biol. Chem.* **238**, 235.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 893.
Sambeth, W., Nesheim, M. C. & Serafin, J. A. (1967). *J. Nutr.* **92**, 479.

Printed in Great Britain