Mechanisms of heat damage in proteins

6. The digestibility of individual amino acids in heated and propionylated proteins*

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1. The digestibilities of protein and amino acids have been estimated by two different techniques: the analysis of faeces (conventional method) and the analysis of ileal contents (ileal technique).

2. Freeze-dried muscle protein was found by both techniques to be almost completely digested. After autoclaving, the digestibility for the same protein was estimated by the conventional and ileal techniques to be 0·65 and 0·57 respectively.

3. Unmodified lactalbumin was found by both techniques to have a digestibility of about 0·90. Propionylation of the lactalbumin reduced digestibility to 0·82 and 0·79 as indicated by faecal analysis and ileal content analysis respectively.

4. In general, the digestibilities of individual amino acids in any one protein sample were rather uniform, and reflected over-all protein digestibility. For each amino acid, digestibility, as determined by both methods, was lower for the modified protein than for the corresponding control protein: estimates based on ileal content analyses were consistently lower than those obtained by conventional analyses. The ileal technique was considered to be both more convenient and meaningful.

5. From the results obtained by the ileal technique it appears that reduced digestibility is an adequate explanation for the reduction found in nutritional value of the autoclaved protein. In contrast, for the propionylated protein, reduced digestibility of lysine is only a partial explanation of the low availability of this amino acid as estimated by chick growth assay.

6. In our experiments we found that the type of dietary protein used did influence the amino acid composition of the ileal contents. This was most marked with the least-digestible protein. These findings do not support the views of Nasset (1962).

The results discussed in the previous paper (Varnish & Carpenter, 1975) indicated that autoclaving chicken muscle severely reduced the availability of its amino acids, although there were few changes in total amino acid contents. In the study to be described in this paper we wanted to determine the extent to which the reduction in nutritional value was due to reduced digestibility of amino acids. The modification of lactalbumin by propionylation appeared to affect the availability of lysine for the growth of chicks to a much greater extent than that of methionine and tryptophan. We again wanted to see whether, for these products, the digestibility values for amino acids (which normally have smaller errors associated with the estimates than those associated with values from growth assays) would throw light on our earlier findings.

The conventional method for determining digestibility is based on measurements of the intake of a nutrient and of the amounts of the same nutrient recovered in the

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faeces. However, it was thought that the existence of a microfloral population in the hind gut might cause a variable and unknown amount of modification to undigested protein residues, which in turn could give misleading results for digestibility when this is measured by faecal analyses. Certainly it had been found that results obtained using caecectomized or germ-free chicks were different from those obtained using conventional chicks, particularly for damaged protein (Levenson & Tennant, 1963; Nesheim & Carpenter, 1967; Salter & Coates, 1971). As nitrogen is absorbed mainly from the jejunum (i.e. that part of the intestine proximal to the ileum) Payne, Combs, Kifer & Snyder (1968) proposed that the analysis of ileal contents, rather than of faeces, might prove to be a more reliable method of assessing protein and amino acid digestibility.

We have used both these methods to determine the digestibility of protein and amino acids for our test materials.

EXPERIMENTAL

Test materials

Details of the four test materials, control lactalbumin (X 901), propionylated lactalbumin (X 900), control chicken muscle (X 902) and autoclaved chicken muscle (X 903) have been given in a previous paper (Varnish & Carpenter, 1975).

Analytical methods

Crude (CP) protein and amino acids. These were determined as described in a previous paper (Varnish & Carpenter, 1975). For the determination of tryptophan in ileal contents by the method of Miller (1967), the quantities taken were one-tenth those originally specified, which necessitated slight practical modifications to the procedure.

Chromic oxide. The method used was a modification by C. K. Milner (personal communication) of the method of Czarnocki, Sibbald & Evans (1961). A portion of test sample containing about 15 mg Cr_2O_3 was weighed onto a filter paper and the paper folded and put into a 300 ml Kjeldahl flask. Conc. nitric acid (10 ml) was added, the flask was left overnight in a fume cupboard and then gently heated to boil off the HNO_3. Heating was continued until the residue in the flask became a charred black crust. After the residue had been allowed to cool, 15 ml digestion mix (Hill & Anderson, 1958) was added and gentle heat applied until the mixture boiled. When the froth had turned yellow and all the black lumps were dissolved, the heat was immediately reduced, so that the contents of the flask were just simmering. (In our experience it was essential that the heating should be reduced immediately.) Gentle heating was continued for a further 25 min and then the flask was allowed to cool. The contents of the flask were washed into a 100 ml volumetric flask and made up to volume with 1·1 M-H_2SO_4. The extinction at 440 nm for the solution was determined (with 1·1 M-H_2SO_4 as the reference solution) after it had been filtered through a Whatman no. 542 filter paper.

The relationship between Cr_2O_3 concentration and extinction was linear and the sample value was calculated relative to the extinction of a single standard of 15 mg Cr_2O_3 digested with 15 ml digestion mixture and made up to 100 ml.

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For faecal and ileal samples, the analysis was done using one-fifth the quantities specified because amounts of the test material available were limited.

Diets

The same experimental diets were used for both studies. The basal diet A contained (g): arachis oil 50, vitamin mix 5; mineral mix 31·3, Terramycin (oxytetracycline) 0·7, choline chloride 1·5, inositol 1·0, $CaCO_3$ 20, $CaHPO_4$ 25, chromium bread 10 (300 g Cr_2O_3/kg; Kane, Jacobson & Moore, 1950) and starch to 1 kg. Diets B, C, D and E each contained one of the test materials added at a level to supply 200 g CP (N × 6·25)/kg. The vitamin and mineral mixes were those described by Carpenter, McDonald & Miller (1972).

Birds

Faecal digestibility studies. Twenty White Link cockerels were reared on a commercial diet until about 7 weeks old. After an overnight fast (no food or water) each chick was operated on, to expose the ureters (Newberne, Laerdal & O'Dell, 1957). Immediately after the operation four birds were allocated at random to each of the five dietary treatments, to provide three final replicates with one additional bird as a replacement in the event of a post-operative problem (there was none). Each bird was individually housed, and the test diet and water were offered *ad lib.* After a 48 h post-operative recovery period each bird was fitted with a plastic tube (40 mm × 15 mm diameter) in contact with the exposed ureters. A balloon was attached to the distal end of the tube for the collection of urine. The faeces were allowed to fall onto a paper tray resting on the bottom of the cage below a galvanized mesh floor.

For the next 4 d individual faecal collections were made from three randomly selected birds per treatment. These were transferred into labelled bottles at 2 h intervals from 09.00 to 24.00 hours, and stored at −15°. Any material contaminated with urine was discarded. Before analysis, the samples were freeze-dried and ground to pass through a 60-mesh sieve.

Ileal digestibility studies. Seventy-two birds were reared to 7 weeks old as described above. For the next 4 d, food was offered for only two periods/d (09.00–11.00 and 15.30–17.30 hours) to train the birds to eat their food rapidly. Water was available *ad lib.* throughout the experiment. The birds were then individually housed and offered the protein-free diet A (without the chromium bread) for the same two feeding periods/d for a further 2 d to remove residual dietary protein from the birds' intestines. During this time, the minimum food intake by any bird in a single 2 h feeding period was 17 g. After an overnight fast twelve birds were allocated at random to each of the five dietary treatments. Each bird was given a 15 g morning feed of the appropriate test diet, which was rapidly eaten by every bird. Using direct intra-cardial injection of Nembutal (Abbot Laboratories Ltd, Queenborough, Kent) to avoid peristaltic movements of the intestinal tract and its contents, half the birds in each treatment group were killed 2·75 h post-cibum, and the other half 3·5 h post-cibum. As each bird was killed, it was dissected immediately, and the ileum located. For this purpose the ileum was defined as extending from Meckel's diverticulum to a point 40 mm

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proximal to the ileo-caecal junction (Payne *et al.* 1968). The ileal contents of three birds given the same test diet and killed after the same period of time were very gently released into a single labelled tube, giving two 'pooled' samples for each period of time for each diet. The times of feeding the individual birds were staggered so that no more than 5 min elapsed from the time when each bird was injected to the time the ileal contents were collected. The samples were freeze-dried and prepared for analysis by the procedure previously described for faecal samples.

Calculations

The digestibility coefficient for CP was calculated using the formula:

$$D = 1 - \frac{x_2 - x_3}{x_1},$$

where x_1 and x_2 are the amounts of CP (g) accompanied by 1 g Cr_2O_3 in the test diet and faeces of one bird given one of the test diets respectively, i.e. CP in sample (g/kg) ÷ Cr_2O_3 in same sample (g/kg); x_3 is the amount of CP (g) accompanied by 1 g Cr_2O_3 in the faeces of a bird given the N-free control diet. (In effect this calculation assumes that metabolic protein (x_3) is proportional to food consumption.)

An exactly comparable procedure was used with ileal contents and was used also to calculate the digestibility for individual amino acids.

The calculation for digestibility coefficients for CP and for each amino acid was done for each individual sample (i.e. on four ileal samples and three faecal samples per dietary treatment) so that the SE of the mean digestibility could be estimated.

It is important to notice that 'digestibility' is calculated by difference from the direct determination of 'indigestibility'. This explains why the lower digestibility values given in Table 2 appear to have comparatively high errors associated with them. When the errors are expressed relative to the coefficients of indigestibility they are similar for each type of material.

RESULTS

All fifteen chicks used for the faecal collections recovered well from surgery, and no problems were encountered in collecting the samples. After the faecal and ileal samples had been freeze-dried and ground, feather contaminants were seen by microscopic examination, and were removed by passing each sample through a fine-mesh sieve.

The amino acid chromatograms of the faecal and ileal samples revealed a 'peak', close to the cysteic acid 'peak', which had not been observed on chromatograms from the test proteins. This was identified as taurine, and was assumed to be derived from taurocholic acid, secreted in the bile. The mean values for amino acid contents for the ileal samples from the chicks receiving the N-free diet are given in Fig. 1.

Table 1 gives the mean values for CP and Cr_2O_3 contents for the test diets and for the ileal and faecal samples. The levels of both CP and Cr_2O_3 for the faecal samples were consistently higher than the corresponding values for ileal samples. The digestibilities of CP and amino acids are given in Table 2; values have been calculated

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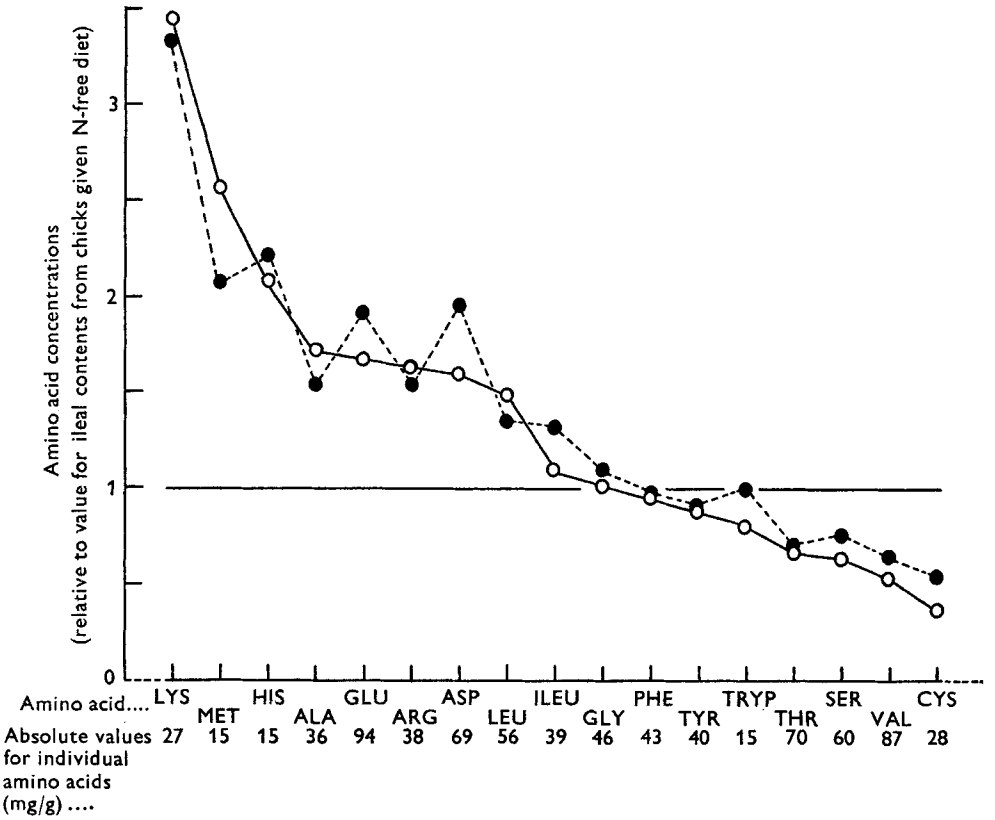

Fig. 1. The relative amino acid concentrations for crude protein (nitrogen × 6·25) of autoclaved chicken muscle (X 903) (○—○) and the ileal contents of chicks receiving X 903 as their dietary protein source (● - - - ●). Each value is expressed relative to the corresponding value for the ileal contents of chicks receiving the N-free diet, for which the absolute values (mg/g) are given. LYS, lysine; MET, methionine; HIS, histidine; ALA, alanine; GLU, glutamic acid; ARG, arginine; ASP, aspartic acid; LEU, leucine; ILEU, isoleucine; GLY, glycine; PHE, phenylalanine; TYR, tyrosine; TRYP, tryptophan; THR, threonine; SER, serine; VAL, valine; CYS, cystine.

from the analysis of both the faeces and ileal contents. Results obtained using both techniques indicated that X 902 (control chicken muscle) had been almost completely digested, and that the digestibility for X 901 (control lactalbumin) was more than 0·90.

Propionylation of lactalbumin had resulted in about a 10% decrease in the digestibility of CP. Although the results obtained using both techniques indicated that autoclaving chicken muscle had substantially reduced the CP digestibility, the 'ileal' digestibility value (0·57) was lower than the 'faecal' digestibility value (0·65).

On the whole, the digestibility for different individual amino acids for any particular sample was rather uniform, and was generally a reflexion of over-all CP digestibility. In every instance the digestibility for each amino acid for the modified protein was less than that for the same amino acid for the corresponding control protein: for the

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Table 1. *The mean values (mg/g) for crude protein (CP; nitrogen × 6·25) content and chromic acid content for the test diets and the air-dried faecal and ileal samples from chicks given these diets*

		Sample					
		Diets		Faeces†		Ileal contents†	
Diet	Protein source*	CP	Cr_2O_3	CP	Cr_2O_3	CP	Cr_2O_3
A	N-free	3	3·07	108	45	68	23
B	Lactalbumin, X 901	208	3·02	290	34	217	25
C	Propionylated lactalbumin, X 900	207	3·04	392	26	322	17
D	Chicken muscle, X 902	203	3·17	201	39	142	27
E	Autoclaved chicken muscle, X 903	204	3·06	557	22	472	14

* Each protein source was included to provide 200 g CP/kg diet; for details of diets, see p. 341.
† Mean values for three different faecal samples and for four ileal samples.

Table 2. *The calculated coefficients of true digestibility for crude protein (nitrogen × 6·25) and individual amino acids for test materials which had been propionylated or autoclaved*, determined by analysis of faeces (FC) or ileal contents (IL)†*

(Mean values for duplicate estimations)

	Lactalbumin				Chicken muscle			
	Control X 901		Propionylated X 900		Control X 902		Autoclaved X 903	
	FC	IL	FC	IL	FC	IL	FC	IL
Crude protein	0·91	0·92	0·82	0·79	0·96	0·98	0·65	0·57
Amino acids								
Aspartic acid	0·93	0·90	0·84	0·71	0·97	0·94	0·64	0·44
Threonine	0·93	0·93	0·84	0·78	0·99	0·94	0·62	0·56
Serine	0·92	0·91	0·82	0·75	0·99	0·99	0·64	0·52
Glutamic acid	0·92	0·88	0·81	0·68	0·98	0·98	0·61	0·47
Glycine	0·89	0·90	0·76	0·76	0·96	0·96	0·51	0·54
Alanine	0·92	0·94	0·85	0·80	0·98	0·98	0·64	0·59
Valine	0·91	0·95	0·84	0·82	0·97	1·02	0·52	0·53
Isoleucine	0·93	0·95	0·89	0·79	0·97	0·99	0·47	0·46
Leucine	0·93	0·93	0·86	0·83	0·98	0·99	0·61	0·58
Tyrosine	0·93	0·94	0·86	0·83	0·98	1·00	0·55	0·56
Phenylalanine	0·94	0·95	0·88	0·88	0·94	1·00	0·58	0·56
Lysine	0·93	0·93	0·82	0·72	0·98	0·99	0·61	0·52
Histidine	0·93	0·95	0·87	0·84	0·97	0·98	0·60	0·50
Arginine	0·93	0·92	0·91	0·84	0·99	0·99	0·66	0·56
Methionine	0·94	0·95	0·88	0·81	0·98	0·99	0·73	0·61
Cystine	0·93	0·93	0·82	0·57	0·99	0·96	0·66	0·47
Tryptophan	0·96	0·96	0·92	0·84	0·96	0·97	0·56	0·47
Over-all mean values:								
FC procedure	0·927	—	0·849	—	0·974	—	0·602	—
IL procedure	—	0·930	—	0·780	—	0·981	—	0·528
Average SE of means	0·017	0·007	0·026	0·028	0·007	0·011	0·063	0·058
Range of SE, calculated for individual amino acids	(0·003–0·027)	(0·002–0·013)	(0·005–0·037)	(0·005–0·074)	(0·000–0·045)	(0·003–0·060)	(0·003–0·106)	(0·037–0·100)

* For details, see Varnish & Carpenter (1975).
† For details of procedures, see pp. 341-2.

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'ileal' digestibility results, these differences consistently reached statistical significance. Further, all the calculations of CP and amino acid digestibilities ranked the four test materials in the same order, i.e. X 902 > X 901 > X 900 > X 903.

DISCUSSION

Non-dietary faecal protein is considered to contain sloughed-off gut lining (produced by the passage of food along the alimentary tract), micro-organisms and intestinal secretions which are, at least in part, influenced by the type and quality of dietary protein (Lyman, 1957; Snook, 1968). However, as Snook & Meyer (1964) have suggested that these secretions are largely reabsorbed, the assumption that endogenous N is proportional to food intake seems a reasonable approximation.

Although estimation of digestibility by the faecal and ileal techniques gave slightly different CP digestibility values for X 901 and X 902 (control proteins) and for X 900 (propionylated lactalbumin), the difference was much greater for the least-well-digested test material, X 903 (autoclaved chicken). The lower value obtained by the ileal technique indicated that more CP was recovered in the ileal contents than in the faeces of birds fed X 903, for an equivalent amount of dietary CP eaten. This is consistent with the suggestion that the microflora in the hind gut produce ammonia by their fermentation of undigested protein, which is then absorbed so that less N is recovered in the faeces (thereby giving a misleading value for undigested N). Clearly if little residual protein reaches this part of the gut (as would be the situation for well-digested proteins) then this 'microflora' effect is restricted. This, of course, explains why differences between the values obtained by faecal and ileal techniques for the comparatively well-digested materials, X 902, X 901 and X 900, were small. Values obtained by some other workers can also be explained on this basis. Thus, Nesheim & Carpenter (1967) measured the apparent digestibilities for control and autoclaved cod samples using normal birds, and caecectomized birds in which fermentation of food residues was presumably reduced. They found that the digestibility for control cod samples is equally high whichever type of bird is used; the digestibility of autoclaved cod samples is lower when the caecectomized birds (0·68) are used than when normal birds are used (0·77). However, in a recent publication, Salter & Fulford (1974) reported that values for digestibilities for amino acids from heat-damaged egg albumin obtained using germ-free and conventional chicks are not very different (0·698 and 0·730 respectively). Also, Erbersdobler & Riedel (1972) did not find any significant difference when heat-damaged soya-bean protein was fed to germ-free and conventional chicks.

In a previous paper (Varnish & Carpenter, 1975) we used growth assays to estimate amino acids available to the chick, for each of the four test materials and these results have been compared with the digestibility values estimated in the present study (Table 3). For sample X 903 (autoclaved chicken muscle), it appears that the 'faecal' digestibility values 'over-estimate' available amino acid contents, in comparison with the values obtained using growth assays. In contrast the 'ileal' digestibility values are in fairly close agreement with the values obtained using growth assays. It is

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tempting to suggest that, in this instance, it is the 'ileal' values which are the 'correct' estimates of digestibility. And this, of course, would suggest that increased resistance to digestion caused by autoclaving the chicken meat was an adequate explanation for the reduced availability, leaving no need to speculate on a possible mechanism for any additional impairment of utilization after digestion. However, this argument would not hold if there were a considerable extent of racemization in the heated protein.

For X 900 (propionylated lactalbumin), reduced digestibility, even when determined by analysis of ileal contents, is not sufficient to account for the greatly decreased 'chick growth assay' value for available lysine. It seems, therefore, that part of the lysine absorbed from X 900 is not available to the animal. Certainly, Bjarnason & Carpenter (1969) found propionyl-lysine *per se* in the urine of rats fed on this compound, which has obviously passed through the kidney unchanged. Even though the chick has a kidney acylase (Leclerc & Benoiton, 1968) which hydrolyses propionyl-lysine in vitro, it seems that in vivo only part of the residues are hydrolysed to release lysine for utilization.

The results of chick growth assays suggested that there was no significant difference between X 900 and X 901 (lactalbumin samples) as sources of available methionine and tryptophan. However, both 'ileal' and 'faecal' digestibility values indicate that the digestibilities of methionine and tryptophan from lactalbumin were significantly reduced by propionylation and these results confirm the conclusions of the microbiological assays described in an earlier paper (Varnish & Carpenter, 1975). It is interesting that the 'chick growth assay' value and 'faecal digestibility' value for tryptophan (when these were expressed relative to the corresponding value for X 901 (control lactalbumin)) are very close, 0·96 and 0·95 respectively. It seems reasonable to propose that the samples do really have different abilities to support growth, even though in the assays these differences are too small to be measured with confidence. The 'ileal' values are again lower than the 'faecal' values.

The ileal technique is easier to use and requires less time than the faecal analysis procedure which, with birds, requires a surgical procedure to allow the separate collection of faeces and urine. Also, we feel that it can be expected to give meaningful estimates of digestibility. However, further work is needed before this method can be generally accepted. Soares & Kifer (1971) found that, although the estimates of amino acid digestibilities obtained by the ileal technique tended to be lower as the quality of dietary fish meal decreased, the range of digestibility did not fully reflect the differences shown by earlier chick growth assays. Bayley, Cho & Holmes (1974) fed pigs different protein meals and collected the ileal digesta over a 9 h period. During this period both the concentration and amino acid composition of the N in the digesta changed, leading the authors to stress the importance of basing analyses on a representative sample of all the digesta flowing through the ileum.

The general pattern of all our results leads us to conclude that autoclaving chicken meat had modified the protein structure in such a way that the enzymic attack necessarily associated with digestion is hindered. None of the experiments described by us gives any indication of the chemical nature of these changes, but Mauron

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Table 3. *The availability of amino acids from proteins which had been propionylated or autoclaved*, expressed relative to the corresponding values for the control proteins, determined by different techniques*

	Lactalbumin Propionylated X 900			Chicken muscle Autoclaved X 903		
	GR	IL	FC	GR	IL	FC
Lysine	0·48	0·77	0·88	0·56	0·52	0·62
Methionine	1·00	0·85	0·94	0·66	0·62	0·74
Tryptophan	0·96	0·88	0·95	0·44	0·48	0·58

GR, chick growth assays (after Varnish & Carpenter, 1975); IL, FC, 'ileal' and 'faecal' digestibilities respectively; for details of procedures, see pp. 341-2.

* For details, see Varnish & Carpenter (1975).

(1972) and Bjarnason & Carpenter (1970) have given their reasons for postulating condensation reactions between the ϵ-amino groups of lysine residues and the carboxyl groups (or more likely, the CO-NH_2 groups) of glutamic and aspartic acids (or glutamine or asparagine, respectively). Certainly one would anticipate reduced enzymic attack when the ϵ-NH_2 groups of lysine in a protein molecule are blocked (cf. review by Spande, Witkop, Degani & Patchornik, 1970). Indeed, the reduced digestibility of X 900, which had a propionyl group at the lysine residues, is an example of this.

Nasset (1962) postulated that dietary protein is diluted by several times as much endogenous protein in the gut, which provides a homoeostatic mechanism preventing wide fluctuations in the amino acid mixture available for absorption. This postulate was based on many experiments (Dreisbach & Nasset, 1954; Nasset & Davenport, 1955; Nasset & Ganapathy, 1962) in which it was consistently found that dietary protein had no influence on intestinal amino acid composition. In contrast, Crompton & Nesheim (1969) reported that amino acid composition of the different parts of the intestine of ducks fed on soya-bean diets generally reflected the amino acid composition of the latter, particularly in the middle intestine. The amino acid composition of the more distal segments of the intestine was more closely allied to that found in birds fed on a N-free diet; the authors therefore concluded that dietary protein was absorbed before endogenous protein. The same workers reported evidence that the dilution of dietary protein by endogenous protein was not large enough to mask the composition of the ingested protein. Mettrick (1970), feeding high-protein diets containing casein or egg albumin to rats, reported that the N and amino acid contents of different regions of the gastrointestinal tract differed significantly depending on the source of dietary protein, and that the molar ratios and concentrations for the amino acids were dependent on the dietary protein source, the region of the intestine studied, and the time after feeding. They concluded that nitrogenous N was insufficient to maintain homoeostasis for the whole intestine. These authors suggested that since Nasset's (1962) work was done using animals which had been fasted for

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24 h before the experiment began, and were slaughtered 1·5 h post-cibum, it may be that the exogenous protein digestion and absorption was already virtually complete by that time, leaving only endogenous protein. Geiger, Human & Middleton (1958), Chen, Rogers & Harper (1962) and Mettrick (1970) also obtained results which led them to question Nasset's (1962) postulation.

In our experiments, reported in this study, we found that the amino acid composition of the ileum contents was greatly modified by the type of dietary protein. Clearly the more indigestible a protein is, the greater the proportion of the dietary intake that is left in the intestine, and the greater the effect of the constituent amino acids on the total amino acid composition. Fig. 1 shows the amino acid levels plotted for X 903 (autoclaved chicken muscle), the ileal contents of birds fed on the N-free diet, or on the diet containing X 903. Clearly the latter values are in close agreement with the values for X 903 itself, and differ considerably from the ileal amino acid composition found for chicks fed on a N-free diet. We must therefore record that our findings do not lend support to the theory of Nasset (1962).

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