Mechanisms of heat damage in proteins

5. The nutritional values of heat-damaged and propionylated proteins as sources of lysine, methionine and tryptophan*

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1. The preparation of a propionylated protein is described, and the effects of this treatment on amino acid composition and availability are compared with the effects of severe heat treatment (autoclaving) of a protein.

2. Using chemical analyses, changes exceeding 5 % for total tyrosine, histidine, methionine and cystine contents were found after propionylation of the protein. Autoclaving of the protein resulted in changes in total serine, lysine, methionine, cystine and tryptophan contents.

3. Microbiological estimates of total amino acid contents were not in close agreement with the chemical estimates for the autoclaved protein.

4. Fluorodinitrobenzene-reactive lysine content was reduced to almost zero by propionylation, and by almost 40 % by autoclaving.

5. Both propionylating and autoclaving protein reduced the amount of lysine available to the chick by about half. In contrast, the availabilities of methionine and tryptophan to the chick were unchanged by propionylation, but were reduced to 0·66 and 0·44 respectively, relative to the untreated protein, by autoclaving.

6. Because of the difficulties of obtaining reliable absolute estimates of amino acid availability using chick growth assays, our interpretation of results is mainly based on relative values. The merits of microbiological estimates of amino acid availability are assessed.

Reports about the damage to proteins heated in the absence of carbohydrate are extensive, and cover a whole range of studies, from chemical to biological (cf. review by Carpenter & Booth, 1973). The results of biological studies have indicated a reduction in the nutritional value of these proteins, and in the utilization of some amino acids. It has been hypothesized that at least some of these findings can be attributed to a reaction which occurs between the ϵ-amino groups of lysine residues and the amide groups of asparagine and glutamine, giving rise to unnatural amide bonds within or between protein chains. Such inter- or intra-molecular cross-linkages might be expected to reduce nutritive value by impeding enzymic attack in vivo.

In this paper we describe the chemical modification of a protein by propionylation of the ϵ-NH_2 groups of lysine residues in lactalbumin, so that it contains unnatural amide bonds of the general type postulated. The nutritional effects of this treatment are compared with the effects of severe heat treatment on proteins. This report is restricted to the estimation of total amino acids by chemical and microbiological techniques, and of available amino acids estimated using fluorodinitrobenzene (FDNB) (lysine only), microbiologically and using chick growth assays. Since chick

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growth assays are used to determine directly the quantity of the limiting amino acid available to the animal, we believe that they represent the most appropriate tests of availability, against which other estimates should be calibrated. The second paper of this series (Varnish & Carpenter, 1975) will be entirely concerned with the estimation of protein and amino acid digestibility, and the extent to which these estimates explain the results presented in the present paper.

EXPERIMENTAL

Test materials

Propionylated lactalbumin (*X* 900). A single batch of lactalbumin (Nutritional Biochemicals Corporation, Cleveland, Ohio, USA) was used to prepare both the control and the propionylated lactalbumin samples.

Propionylation was done using a method comparable to that of Fraenkel-Conrat, Bean & Lineweaver (1949) and method 2 of Bjarnason & Carpenter (1969). To an ice-cold aqueous solution of sodium propionate (300 g/l) was added 100 g lactalbumin/l and these were thoroughly mixed. Propionic anhydride (1 ml/g protein) was added slowly for a period of 1 h, during which the mixture was stirred continuously and maintained in an ice bath. After a further 1 h period the mixture which was stirred continuously was equilibrated to room temperature. The protein was precipitated by the addition of ethanol, and was left overnight to settle. The supernatant fraction was decanted off and discarded. The precipitate was centrifuged, resuspended in water, reprecipitated with ethanol, and centrifuged; this washing procedure was repeated three times. The material was roughly ground once before it had equilibrated with the atmosphere, was air-dried at room temperature, and finally ground to a fine powder.

Control lactalbumin (*X*901). Control material was prepared using the propionylation procedure, except that propionic anhydride was not added.

Control chicken muscle (*X* 902). This was an untreated commercial batch of freeze-dried, white chicken muscle (Batchelors Foods Ltd, Ashford, Kent), milled to a fine powder.

Autoclaved chicken muscle (*X* 903). A portion of X 902 containing 140 mg moisture/g was packed into tins, 80 mm deep and 100 mm diameter, which were sealed under nitrogen gas. The tins were then autoclaved together for 27 h at 116°. This treatment was reported by Miller, Carpenter & Milner (1965) to cause severe heat damage to cod muscle.

All test materials were packaged in air-tight containers, and stored at −15°.

Propionyl-lysine. The ϵ-N-propionyl-L-lysine used was part of the batch made by Bjarnason & Carpenter (1969).

Analytical methods

Crude protein content. N content was determined using macro-Kjeldahl digestion (Association of Official Agricultural Chemists, 1965) with a potassium salt catalyst (added as Kjeltabs 'C'; Thompson & Capper Ltd, Liverpool). The ammonia produced was distilled into boric acid solution (10 g/l) containing a mixed indicator, for

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titration with 0·014 M-HCl (Ma & Zuazaga, 1942). Crude protein content was expressed as g N × 6·25/kg.

Total amino acid contents. Duplicate samples for each of the four test materials were acid-hydrolysed using the method of Weidner & Eggum (1966). The acid-hydrolysates were prepared and analysed using an AutoAnalyzer (Technicon Instruments Co. Ltd, Basingstoke, Hants), by the method described by Technicon Instruments Corporation (1963), with norleucine as the internal standard.

For the sulphur amino acids, preliminary quantitative oxidation was done using the method of Weidner & Eggum (1966). The oxidized proteins, with methionine converted to methionine sulphone (MS) and 'cystine + cysteine' converted to cysteic acid (CA), were then acid-hydrolysed and prepared as described previously.

Using the standard column conditions (Technicon Instruments Corporation, 1963), or the short column systems described by Thomas (1965), the resolution of aspartic acid and MS was inadequate. Adjustment of the pH of the hydrolysate to 1·9 (Hartley, 1966) before using the analytical system of Thomas (1965) did not produce resolution that was good enough for accurate determination. A. W. Hartley (personal communication) suggested that the salt concentration of the acid-hydrolysates might have been too high, and adequate resolution was achieved when the pH of buffer in the first chamber of the 'autograd' was adjusted with citric acid instead of HCl. Further improvements were obtained by applying the oxidized acid-hydrolysates to the column at pH 2·0, and 'washing in' with 0·5 ml 'pH 2·0' buffer, made up using only citric acid.

For the estimation of total tryptophan content the method of Miller (1967) was used. FDNB-reactive lysine content was determined by the direct method of Carpenter (1960).

Microbiological assays. Total and available amino acid contents were determined by the method of Ford (1962) except that the nucleotide concentrations in the assay media were doubled as suggested by Kennedy (1965). The extinction at 580 nm, which was used to estimate the growth response, was measured using a colorimeter (Lumetron Model 400A; Photovolt Corporation, New York, USA).

Biological assays. The chicks used for all assays were White Link cockerels (Sterling Chicks Ltd, Welwyn Garden City, Herts.). Their management, the general experimental design, and procedural and statistical methods were the same as those described by Carpenter, March, Milner & Campbell (1963). For each assay the following experimental diets were fed to four replicate groups each containing three chicks: (1) the unsupplemented basal diet deficient in the amino acid to be assayed; (2) the basal diet supplemented with three graded levels of the limiting amino acid (supplied by Koch-Light Laboratories, Colnbrook, Bucks.), to obtain the standard response; (3) the basal diet supplemented with two levels of each test protein. Food conversion efficiency (FCE) (g weight gain/g food intake) was estimated for each diet. Correction was made for spilt food and the result for any group in which a chick died or suffered injury was discarded.

Available lysine content. In a preliminary experiment, the FCE for the unsupplemented basal diet used by Carpenter *et al.* (1963) was 0·434, and the range of the

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Table 1. *The composition of the experimental diets (g/kg) used in chick growth assays for three amino acids, lysine, methionine and tryptophan*

Assay ...	Lysine	Methionine	Tryptophan
Ingredient			
Groundnut meal	—	346	—
Autoclaved groundnut meal*	To supply 200 g CP/kg	—	—
Dried whey	—	50	25
Maize-gluten feed	—	—	To supply 170 g CP/kg
Whole wheat	200	—	—
Dried-grass meal	20	—	—
Arachis oil	100	50	20
Terramycin 5†	0·7	—	0·7
Choline chloride	1·5	5	—
myo-Inositol	1·0	1	—
Vitamin mix‡	5	5	5
Mineral mix‡	31·3	31·3	31·3
$CaCO_3$	20	20	20
$CaHPO_4.2H_2O$	25	25	25
L-lysine HCl	—	4	3·6
Cystine	—	2	—
Glycine	—	2	—
L-threonine	2·4	2·4	—
DL-methionine	3·2	—	2·4
Gelatin‡	—	—	85
Zein‡	36	—	—
Glycine–glutamic acid (1:5·9) mix‡	—	27	—
Oil–sawdust mix§	—	—	to 1000
Starch	to 1000	to 1000	—

CP, crude protein (nitrogen ×6·25).
* For details of preparation, see below.
† Containing 22 mg oxytetracycline/g (Pfizer Ltd, Folkestone, Kent).
‡ Levels were reduced so that all diets within each assay were isonitrogenous when test materials were added.
§ As described by Carpenter, McDonald & Miller (1972).

standard response, for diets supplemented with 0–4·8 g lysine equivalent/kg, was only 0·215. This suggested that our basal diet in this experiment was not sufficiently deficient in available lysine and only a small part of the linear response range to lysine was being used.

The modified basal diet, detailed in Table 1, contained decorticated, extracted groundnut meal (100 mg moisture/g), which had been autoclaved for 4 h at 121°. It had been found previously that this product, when supplemented with methionine and threonine, gave a large response to additional lysine (Anantharaman & Carpenter, 1971). In the first assay using the modified diet (Expt 140) the groundnut meal had been autoclaved in 254 mm × 254 mm tins, and although the material on the outside of the autoclaved sample had darkened in colour, the innermost material was light in colour and apparently undamaged. This was confirmed when the mixed material was used in the diets, as the FCE for the unsupplemented basal diet was 0·321, and the upper limit of the standard response range was beyond the linear response range.

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In the subsequent assay (Expt 144) another portion of the same groundnut meal (containing 100 mg moisture/g) was autoclaved using a number of aluminium-foil envelopes, each 350 mm × 350 mm and no more than 25 mm thick. An extra 15 min was added to the period of autoclaving so that the contents of the envelopes reached 121°. The packs were left to cool before they were opened. Any hard, damp lumps, where water had permeated the envelopes, were discarded, and the remainder was found to be uniformly deepened in colour. The material was ground, sieved and mixed before it was used in the diets. In this experiment, the FCE for the unsupplemented basal diet was only 0·113 and the range of the standard response was increased to 0·384. A basal diet prepared in this way was then used to assay the test materials (Expt 145).

Because the total lysine content of all test materials was similar, it was convenient to include them in the diets to contribute the same two levels of crude protein, (14·5 and 29 g/kg). The diets were kept isonitrogenous by adjusting the level of zein. The standard response was obtained by supplementing the basal diet with 2, 4 and 6 g lysine HCl/kg (1·6, 3·2 and 4·8 g lysine/kg respectively). For the final 2 d before they were divided randomly into groups the chicks were fed on a low-lysine diet (the basal diet of Carpenter *et al.* (1963) supplemented with 35 g fish meal/kg) instead of a commercial feed.

Twelve of the fourteen diets used in Expt 149 were the same as those used in Expt 145, and the other two diets contained 2·21 and 4·42 g propionyl-lysine/kg respectively (i.e. chemically equivalent to 1·6 and 3·2 g lysine/kg respectively).

Available methionine content. The basal diet used has been described by Varnish (1971) and was referred to as diet 1 by Carpenter, McDonald & Miller (1972). In Expts 147 and 150, the standard response was obtained by supplementing the basal diet with 0·2, 0·4 and 0·6 g methionine/kg. All test materials were assayed at two dietary levels (8·84 and 16·88 g 'test material' crude protein/kg diet respectively).

Available tryptophan content. The basal diet used was that described by Harwood & Shrimpton (1969). The two control proteins, whose tryptophan contents differed considerably, were added to the basal diet to provide (g crude protein/kg): lactalbumin 10 and 20, chicken muscle 20 and 40. The standard response was obtained by supplementing the basal diet with 0·2, 0·4 and 0·6 g tryptophan/kg. For all tryptophan assays (Expts 152, 155, 159), the chicks received a commercial diet in the pre-experimental period.

RESULTS

Total amino acids

The results of the crude protein estimations, and chemical amino acid analyses for acid-hydrolysates of the test materials are given in Table 2; the percentage change in the values for the modified proteins, relative to those for the control protein is given, where it exceeds 5%.

The results for FDNB-reactive lysine indicated that the propionylation technique had been successful in blocking almost all the ϵ-NH_2 groups of the lysine residues in lactalbumin. Autoclaving chicken muscle reduced FDNB-reactive lysine by 38%.

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Table 2. *Crude protein (nitrogen × 6·25) (mg/g) and fluorodinitrobenzene (FDNB)-reactive lysine and chemically determined total amino acid (mg/g crude protein) contents for protein samples which had been propionylated or autoclaved**

(Mean values for duplicate determinations. Values in parentheses refer to the percentage change in value for modified protein relative to control protein where it exceeds 5 %)

	Lactalbumin		Chicken muscle	
	Control X 901	Propionylated X 900	Control X 902	Autoclaved X 903
Crude protein	719	706	885	793
FDNB-reactive lysine	103	4 (−96)	89	55 (−38)
Total amino acids:				
Aspartic acid	136	133	107	112
Threonine	55	57	45	45
Serine	49	49	41	38 (−7)
Glutamic acid	179	186	162	158
Glycine	23	24	45	47
Alanine	56	56	58	62
Valine	63	64	44	45
Isoleucine	61	61	43	43
Leucine	131	135	79	83
Tyrosine	42	46 (+7)	34	35
Phenylalanine	42	44	39	41
Lysine	103	102	98	92 (−6)
Histidine	22	24 (+10)	31	31
Arginine	35	35	58	61
Methionine	32	30 (−8)	36	38 (+6)
Cystine	46	36 (−22)	13	10 (−19)
Tryptophan	25	24	14	12 (−11)

* For details of procedures, see p. 326.

Comparison of the values for X 900 and X 901 (control and propionylated lactalbumin samples) indicated that propionylation had had little affect on the total amino acid contents except for the apparent increases in tyrosine and histidine contents, and decreases in cystine and methionine contents. Autoclaving chicken muscle had apparently caused partial destruction of serine, lysine, cystine and tryptophan, with a slight increase in methionine content.

The microbiological estimates of total amino acid contents are given in Table 3. For both X 900 and X 901 and for X 902 (control chicken muscle) the values were generally within 10 % of the corresponding chemical estimates (Table 1), except for isoleucine content for X 902, which was about 20 % lower than the chemical value. However, for X 903 (autoclaved chicken muscle) there were considerable discrepancies between the microbiological and chemical estimates for total amino acids; microbiological values for lysine, arginine and methionine were 13, 26 and 33 % lower than the two corresponding chemical estimates respectively. Microbiological estimates of isoleucine were consistently higher than the corresponding chemical estimates.

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Table 3. *Total and available amino acid contents (mg/g crude protein) for protein samples which had been propionylated or autoclaved*, determined microbiologically, using* Streptococcus zymogenes

(Mean values with their standard errors; no. of replicates in parentheses)

	Lactalbumin				Chicken muscle			
	Control X 901		Propionylated X 900		Control X 902		Autoclaved X 903	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Methionine								
Total	28·8	0·8(5)	29·3	0·6(6)	31·5	0·7(6)	25·8	0·7(6)
Available	21·7	0·4(6)	20·0	0·3(6)	30·3	0·6(6)	11·7	0·3(6)
Tryptophan								
Available	14·8	0·3(7)	14·1	0·6(8)	11·6	0·5(10)	3·3	0·5(8)
Leucine								
Total	132·5	1·3(4)	127·0	2·0(4)	83·0	2·0(2)	72·0	0·9(4)
Available	100·5	0·5(2)	91·0	0 (2)	66·0	0 (2)	29·0	1·0(2)
Isoleucine								
Total	67·0	0 (2)	64·8	1·0(4)	53·0	1·0(2)	49·0	1·2(4)
Available	47·0	0 (2)	44·5	0·5(2)	50·5	0·5(2)	15·4	1·1(2)
Arginine								
Total	34·0	1·2(3)	36·3	1·3(3)	56·0	0 (2)	45·5	1·5(2)
Available	31·5	0·5(2)	32·5	0·5(2)	58·0	0 (2)	26·0	0 (2)
Histidine								
Available	19·5	0·2(2)	17·1	0·5(2)	29·8	0·5(2)	11·0	0·8(2)

* For details of procedures, see p. 327.

Available amino acids

The results of the chick growth assays for available lysine, methionine and tryptophan contents of the test materials, which were statistically valid, are given in Table 4. The relative potency of propionyl-lysine was estimated (Expt 149) to be 72 % (range 67–77) that of pure lysine.

Lysine. The lysine potency values obtained from Expt 149 were consistently higher than those from Expt 145 although for the two control materials (i.e. the materials with the highest absolute estimates) the percentage differences were quite small. The range of the fiducial limits was similar for all estimates in both assays, i.e. approximately 20 mg lysine/g crude protein and the coefficients of variation range from 0·04 to 0·10.

It is clear from both lysine assays (Expts 145 and 149) that propionylating lactalbumin and autoclaving chicken muscle had, in each instance, reduced the availability of lysine for the chick by approximately half.

Methionine. There was good agreement between the estimates of methionine potency for the test materials obtained from the two chick growth assays (Expts 147 and 150), although the coefficients of variation for Expt 150 were lower. Generally, the coefficients of variation for the methionine estimates were not as low as those for the lysine estimates, which is probably explained by the range of FCE response, which was smaller for the 'methionine' assay.

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Table 4. *Potencies for protein samples which had been propionylated or autoclaved* (mg/g crude protein (nitrogen × 6·25)), as sources of three amino acids, determined using chick growth assays*

		Lactalbumin				Chicken muscle			
		Control X 901		Propionylated X 900		Control X 902		Autoclaved X 903	
Amino acid	Expt no.†	Estimate‡	CV	Estimate‡	CV	Estimate‡	CV	Estimate‡	CV
Lysine	145	121(110–131)	0·04	55(44–65)	0·10	109(99–117)	0·05	55(45–65)	0·10
	149	129(119–140)	0·04	66(56–76)	0·08	114(104–124)	0·05	69(59–79)	0·07
Average		125		60·5 (0·48)§		115		62 (0·56)§	
Methionine	147	25(20–31)	0·11	25(19–31)	0·11	29(23–36)	0·11	19(13–25)	0·16
	150	26(22–31)	0·09	26(23–30)	0·08	27(23–31)	0·08	18(14–21)	0·10
Average		25·5		25·5 (1·00)§		28		18·5 (0·66)§	
Tryptophan	146	27(23–32)	0·08	26(21–31) (0·96)§	0·10	16(14–19)	0·08	7(5–10) (0·44)§	0·18

CV, coefficient of variation expressed as a proportion of the estimate.
* For details of procedures, see p. 326.
† For details, see pp. 327–9.
‡ Each value is given with the 0·95 fiducial limits.
§ Value in parentheses is the average expressed as a proportion of the control value.

Propionylation of lactalbumin had no effect on the availability of methionine to the chick. In contrast, autoclaving chicken muscle reduced by one-third the amount of methionine available to the chick.

Tryptophan. Using FCE as the measurement of response in the chick growth assay (Expt 146), statistical analysis indicated very highly significant blank and intersection values which, judging from visual examination of the results, were probably caused by the pattern of responses to autoclaved chicken muscle. The results were re-analysed on a weight-gain basis and when the blanks were omitted, the statistically valid estimates given in Table 3 were obtained.

In three further experiments (Expts 152, 155 and 159) the standard responses showed significant curvature, indicating failure of a test for validity of a slope-ratio assay (Finney, 1974). The diet containing maize gluten used in Expt 152 was from the same batch as that used in Expt 146, and that used in Expt 155 was again from another batch which had been used successfully for this assay in another laboratory. We cannot explain the problems encountered in our laboratory.

From the statistically valid estimates obtained in Expt 146, it appeared that tryptophan, like methionine, was unaffected by propionylating lactalbumin but reduced by more than 50% by autoclaving chicken muscle. (The estimates based on FCE, although not statistically valid, did agree closely with the statistically valid estimates based on weight gain.)

The microbiological assays gave lower absolute estimates for available tryptophan content than did the chick growth assays for each test material, as they did for methionine in X 900, X 901 and X 903. However, the results of microbiological assays agreed

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Table 5. *Total and available amino acid contents for protein samples which had been propionylated or autoclaved*, relative to the corresponding values for the control proteins*

Amino acid	Method of determination†	Lactalbumin Propionylated X 900 Total	Lactalbumin Propionylated X 900 Available	Chicken muscle Autoclaved X 903 Total	Chicken muscle Autoclaved X 903 Available
Lysine					
	Chemical	0·99	0·04	0·94	0·62
	Chick growth assay	—	0·48	—	0·56
Methionine					
	Chemical	0·92	—	1·07	—
	Streptococcus zymogenes	1·00	0·92	0·82	0·39
	Chick growth assay	—	1·00	—	0·66
Tryptophan					
	Chemical	0·96	—	0·89	—
	Strep. zymogenes	—	0·95	—	0·28
	Chick growth assay	—	0·96	—	0·44
Leucine					
	Chemical	1·03	—	1·05	—
	Strep. zymogenes	0·96	0·91	0·87	0·44

* For details of procedures, see p. 329.
† For details, see 'Experimental' section.

with those for the chick growth assays in indicating that the effects of propionylation on available methionine and tryptophan contents were small. The results also indicated a large reduction in available methionine and tryptophan contents for X 903, even greater than that indicated by the chick assays (Table 5).

Comparing the results for total and available amino acid contents for all four test materials (Table 3), the highest extent of availability for *Streptococcus zymogenes* was found in X 902 (control chicken muscle), and the lowest was in X 903. The results for X 900 and X 901 were between those for X 902 and X 903, with the results for the control material being, in general, marginally higher than those for the propionylated product.

DISCUSSION

Total amino acids

Looking first at the chemically determined results, the partial destruction of tryptophan in X 903 (autoclaved chicken muscle), may be apparent rather than real, since the absolute values were so low. Certainly Miller, Carpenter, Morgan & Boyne (1965) reported no tryptophan destruction in autoclaved cod, although other groups including Mason & Weidner (1964) and Donoso, Lewis, Miller & Payne (1962) have found losses after heat treatments.

The small reduction in total lysine content for X 903 confirms the findings of Bjarnason & Carpenter (1970) working with heat-treated bovine plasma albumin (BPA) and Miller, Carpenter & Milner (1965) working with autoclaved cod. Both these groups reported considerably higher percentage destruction of 'cystine+cysteine' than that found in our samples. This might be explained by different cystine:

cysteine ratios for the various test proteins, as cysteine is less sensitive to heat destruction than cystine (cf. Bjarnason & Carpenter, 1970).

Some of the apparent increases in amino acid levels as a result of heat treatment must result, at least in part, from loss of N in a volatile form such as NH_3. A 14% increase in methionine content was recorded for autoclaved BPA, but its significance was questioned by the authors (Bjarnason & Carpenter, 1970). The apparent increase in histidine content for propionylated lactalbumin may be an artifact associated with a base-line increase after the NH_3 peak in the chromatogram, which was found consistently in the study. For most amino acids, the chemical results indicated little change in total values for either of the modified proteins.

It has already been noted that the microbiologically determined estimates of total amino acid contents for the test materials agreed reasonably well with the corresponding chemically determined estimates, except those for X 903 where the microbiologically determined values were considerably lower. This suggested that although the 'mild' digestion procedure used for our microbiological assays hydrolysed X 902, X 900 and X 901, the conditions were inadequate for complete hydrolysis of X 903 which has been reported by Otterburn & Sinclair (1973) to be particularly resistant to acid-hydrolysis. It may be that the stronger hydrolysis conditions recommended by Henry & Ford (1965) would result in higher estimates of total amino acid contents for X 903. Certainly, our microbiologically determined estimates gave a misleading indication of considerable amino acid destruction.

Assays for available amino acids

In our chick growth assays, the experimental diets were isonitrogenous within each experiment. Using unbalanced proteins like zein and gelatin for this purpose is not entirely satisfactory, but it seemed preferable to make this correction rather than to make no correction.

Results of biological assays must, of course, be statistically valid, but it is equally important that the confidence limits to the estimates should be reasonably narrow, and the precision of the estimates from our present chick assays was generally better than that for the assays reported by Carpenter *et al.* (1972).

For the chick growth assays for lysine (Expts 145 and 149), the absolute estimates of available lysine content were lower for the modified samples than those for the appropriate controls, and the coefficients of variation were correspondingly higher. Presumably they could be reduced by assaying the modified proteins at higher dose levels so that the responses were nearer the upper limit of the linear response range. However, we were trying to compare directly the potency of each control material with that of its modified counterpart, and it was considered to be most important to compare each pair of materials at equivalent total protein levels.

Another general problem in biological assays is that of systematic errors. For the control materials X 901 and X 902 the estimated assay values for lysine and tryptophan content were, in each instance, greater than the corresponding chemically determined total values, i.e. their availability apparently exceeded 100%. This has been reported previously for some freeze-dried proteins (Miller, Carpenter & Milner, 1965; Atkinson

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& Carpenter, 1970). With these examples the over-estimation in the biological assays is clear from the results, but other results obtained in these and other assays, though not exceeding 100% availability, may still conceal an 'over-estimate'.

Two possible explanations can be advanced. First, the supplementary amino acid may be absorbed more quickly when supplied in the crystalline form rather than as a part of a protein (Rolls, Williams & Porter, 1969), so that it passes through the portal blood to the anabolic sites before the necessary array of other amino acids is present, and is used less efficiently. While this might apply to animals which are fed only at considerable intervals, it is unlikely to apply in assays where the birds have unrestricted access to food. Alternatively the apparent advantage of lysine in peptide chains may be related to findings that these are normally hydrolysed to dipeptides which are absorbed from the intestine in this form. Glycyl-L-lysine is transmitted intact from the intestinal lumen, but does not pass across the mucosa into the bloodstream intact, and it is suggested that different dipeptides are handled differently. It is easy to appreciate how this confuses the situation in relation to biological assays, when unit amino acids are fed in the 'standard-response' diets, and compared to protein-supplemented diets in which the protein is hydrolysed to a mixture of peptides and amino acids.

Over all, it was concluded that the lysine and methionine assay procedures used in this study were reasonably satisfactory for giving relative values, although the preparation of autoclaved groundnut meal for the lysine assay was time-consuming. We were less satisfied with the tryptophan assays.

Utilization of modified proteins

The chick growth assay (Expt 149), gave a value for the lysine potency of crystalline ϵ-N-propionyl-lysine of 72% that for pure lysine. With rats, the same compound was not found to be active (Bjarnason & Carpenter, 1969). This species difference can be explained by the chick having a kidney enzyme, ϵ-N-lysine acylase, which can hydrolyse the propionyl derivative (Leclerc & Benoiton, 1968). This is, therefore, an example of a lysine derivative which can show biological activity for the chick even though it is unreactive to FDNB at the ϵ-N position.

The results given in Table 4 indicate, however, that propionylation of the lysine units in lactalbumin had reduced its value as a source of lysine for the chick to 48% that of the unmodified protein, although there was little change in the value of the protein as a source of methionine or tryptophan. Possibly the propionyl groups have little effect on the general splitting of the protein chains in the digestive tract but hinder specifically the final splitting of lysyl dipeptides. This is considered further in a following paper dealing with digestibility at the ileal level (Varnish & Carpenter, 1975).

Turning to the results of the microbiological assays, it is interesting that even for X 900 (control lactalbumin sample) only arginine was almost completely available, most of the values for amino acid contents suggested 70–75% availability. Similar results were reported by Ford (1962) for freeze-dried, skim-milk powder, with arginine showing maximum availability, and 70–80% availabilities for methionine, leucine and

isoleucine. The microbiologically determined availabilities for methionine, isoleucine, histidine and arginine from X 902 were almost 100%, but that for tryptophan was 87% and for leucine was about 80%.

In contrast to the apparently limited effects of propionylation, results shown in Table 4 indicated that autoclaving chicken muscle substantially reduced the availability of lysine, methionine and tryptophan for the chick. That the availability of methionine was less affected than that of lysine is in agreement with the work of Miller, Carpenter, Morgan & Boyne (1965).

This general effect, that the amino acids in severely heated material are less available to the chick, is in line also with the microbiologically determined results (Table 3). These in turn confirm reports by Dvorak (1968) working with autoclaved BPA, and Ford & Salter (1966).

In agreement with the work of Atkinson & Carpenter (1970), *Strep. zymogenes* assays gave considerably lower available tryptophan values for all four test materials than the corresponding values obtained by chick growth assays. For X 900 and X 901 the relative microbiologically determined values were, however, comparable to the relative values obtained using chick growth assays, as shown in Table 5, although for X 902 and X 903 samples the results from *Strep. zymogenes* assays exaggerated the deleterious effects of autoclaving for both tryptophan and methionine. In absolute terms, the microbiologically determined methionine value was slightly higher than the value from chick growth assay for X 902 (control chicken muscle) but lower for the other samples, particularly X 903 (autoclaved chicken muscle).

Since the microbiological availability of amino acids with unreactive paraffin side-chains, like leucine, has also been reduced by autoclaving chicken muscle, this suggests that the heat treatment has had some general effect on the protein, which prevents the constituent amino acids from being utilized as well as the same amino acids from the untreated control material. Mecham & Olcott (1947) suggested cross-link bond formations between the ϵ-NH_2 groups of lysine and the carboxylic acid groups of glutamic and aspartic acids (or more likely their amide derivatives) in heat-damaged proteins, and subsequently Bjarnason & Carpenter (1970) have done chemical experiments to test this theory; Mauron (1972) has also suggested other bond formations. It seems reasonable to suppose that such reactions would hinder enzymic attack in vivo. We were therefore interested to study the effects of the modification of our test proteins on their digestion by chicks, and experiments to this end are reported in the subsequent paper.

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