Mechanisms of heat damage in proteins

7. The significance of lysine-containing isopeptides and of lanthionine in heated proteins*

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1. Studies have been made with solvent-extracted chicken muscle, bovine plasma albumin (BPA) and other proteins, all severely heated in the absence of carbohydrates so as to cause a large decrease in their fluorodinitrobenzene (FDNB)-reactive lysine contents.

2. ε-N-(β-L-aspartyl)-L-lysine and ε-N-(γ-L-glutamyl)-L-lysine isopeptides were determined after enzymic digestion of heated chicken muscle, and their content was found to increase as the material was subjected to more heat treatment. Heated chicken muscle was not found to contain lanthionine. Heated BPA, on the other hand, was found to contain lanthionine but not the isopeptides. Both lanthionine and isopeptide cross-linkages were detected in most of the other heated proteins. There was some difficulty in quantifying the amounts of isopeptides formed on heat treatment, because the enzymic digestion procedure used in their isolation appeared to be incomplete. Neither lysinoalanine nor ornithinoalanine was detected in any of the test materials.

3. The severely heated chicken muscle was fed to rats, and ileal and faecal digestibilities were studied. Protein digestibility was found to be greatly reduced after heat treatment, although the isopeptides themselves appeared to be at least as digestible as the total N component, total lysine, or FDNB-reactive lysine. However, the reduction in ileal N digestibility only partly accounted for the much larger reduction in nutritive value, as measured by net protein ratio ((weight loss of N-free animals + weight gain of test animals) ÷ weight of crude protein (N × 6·25) consumed by test animals). Possible reasons for this are discussed.

It is well known that when proteins, even in the absence of carbohydrates, are heated under really severe or prolonged conditions their nutritional value is reduced. However, the chemical mechanisms of this heat damage and its nutritional consequences are not fully understood. It is believed that heat causes the formation of new enzyme-resistant linkages within the protein molecule, so reducing its digestibility and the biological availability of some of the constituent amino acids (e.g. Mauron, 1972; Ford, 1973). There are many possible cross-linking reactions, but one of the most likely seems to be the formation of new isopeptide bonds by reaction of the ε-amino group of lysine with either the carboxyl group of aspartic or glutamic acids (Mecham & Olcott, 1947; Asquith & Otterburn, 1969), or more probably with the amide groups of glutamine and asparagine (Bjarnason & Carpenter, 1970). The term isopeptide is used to differentiate these bonds from the normal peptide link.

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Other cross-linkages may result from the degradation of cystine. Heat can cause fission of the disulphide bond, yielding dehydroalanine which may condense with cysteine to form lanthionine, or with the ε-amino group of lysine to form lysinoalanine (Horn, Jones & Ringel, 1941; Bohak, 1964; Ziegler, 1964). These two compounds may be estimated after acid-hydrolysis, but the lysine-isopeptides are acid-labile and have been determined as residues after enzymic digestion of the ordinary peptide links (Pisano, Finlayson & Peyton, 1969; Asquith, Otterburn, Buchanan, Cole, Fletcher & Gardner, 1970; Asquith, Otterburn & Gardner, 1971).

In this investigation the formation of these cross-linkages in heated proteins, and the fate of the lysine-containing isopeptides in the rat's digestive tract have been studied.

EXPERIMENTAL

Test materials

Chicken muscle, X 902, was obtained from Batchelor's Foods Ltd, Ashford, Kent; it consisted of freeze-dried white muscle which we milled into a fine powder (Hurrell & Carpenter, 1974). Fat was extracted using a Soxhlet apparatus, refluxing for 24 h with chloroform–hexane (3:1 v/v), then for 3 h with diethyl ether which was removed by leaving the material spread out overnight at 27°. The extracted material, X 902E, was adjusted to 150 mg moisture/g and packed into glass ampoules, as described by Carpenter, Morgan, Lea & Parr (1962). These were flushed with nitrogen before sealing and then autoclaved for different periods. For the animal assay, the same material was autoclaved for 8 and 27 h in sealed McCartney bottles. Chicken muscle, X 903, was the material used by Varnish & Carpenter (1975*a*, *b*), and had been stored under N_2 at −20°. It had been prepared from X 902 which had been adjusted to 140 mg moisture/g and sealed under N_2 into small cans (80 mm deep, 100 mm diameter), which were then autoclaved for 27 h at 116°. It was not solvent-extracted.

Bovine plasma albumin (BPA), which was either unheated or heated for 8 and 27 h at 121°, and the blood meals, X 949 and X 953, dried at low and high temperatures respectively, were the materials described in a previous paper (Hurrell & Carpenter, 1975).

Zein, egg albumin, casein and lactalbumin samples were the same materials as those used by Bjarnason & Carpenter (1970).

Analytical procedures

N, total lysine, fluorodinitrobenzene (FDNB)-reactive lysine by the 'difference' method, and FDNB-reactive lysine by the 'direct' method were determined as described previously (Hurrell & Carpenter, 1974). Chromic oxide was determined using the procedure described by Varnish & Carpenter (1975*b*), scaled down five times because of the limited amount of ileal and faecal materials.

ε-N-(γ-L-glutamyl)-L-lysine and ε-N-(β-L-aspartyl)-L-lysine. The enzymic digestion procedure of Cole, Fletcher, Gardner & Corfield (1971) was followed by separation and measurement of the isopeptides by the method of Spackman, Stein & Moore (1958), using an AutoAnalyzer (Technicon Instruments Co. Ltd, Basingstoke, Hants)

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with a 1400 mm column and a '6 h' buffer gradient. Lithium salts were used in the buffer system (Sinclair & Otterburn, unpublished results) and the flow-rate was 1·0 ml/min. As aspartyl-lysine is eluted at the same time as norleucine, L-leucyl-glycine was used as an internal standard. Total amino acids were measured, after acid-hydrolysis, by the same procedure. The recovery of free glutamyl-lysine added at the beginning of the enzymic digestion of heated X 902E (with thymol present to prevent bacterial action) was 95%. A recovery factor of 95% has been used when calculating the amounts of glutamyl- and aspartyl-lysine recovered from enzymic digests of all heated proteins. The recoveries of free glutamyl-lysine added to enzymic digests of the ileal and faecal contents were 87 and 66% respectively, and these have been similarly applied in the calculations.

Lanthionine. This amino acid was estimated after acid-hydrolysis by the paper chromatographic method of Dowling & Crewther (1964). The colour was developed using Chinard's reagent (Chinard, 1951).

Lysinoalanine and ornithinoalanine. These were determined in acid-hydrolysates by an electrophoretic method (Asquith & Garcia-Dominguez, 1968), followed by a colorimetric reaction with a cadmium–ninhydrin solution (Heilman, Barollier & Watzke, 1957).

Animal experiments

Three samples of X 902E (unheated, and heated for 8 or 27 h at 121°) were assayed for their value as the sole protein source for young rats using the net protein ratio (NPR) test (Bender & Doell, 1957), and for the faecal and ileal digestibilities of the N and the different lysine components.

Sixteen 21-d-old female rats of CFY strain (Anglia Laboratory Animals, Huntingdon, Cambs.) were randomly allocated to eight cages so that each contained two rats, and two cages of rats were allotted to each of the four diets. The N-free diet consisted of (g/kg): sucrose 850, cellulose powder 40, maize oil 50, mineral mix 40, vitamin mix 10, chromium bread 10. The three test diets contained X 902E, unheated or heated for 8 or 27 h at 121°, added to the N-free diet at a level of 100 g crude protein ($N \times 6{\cdot}25$)/kg, at the expense of sucrose. The mineral and vitamin mixes were those used by Waibel & Carpenter (1972), and the Cr bread contained 700 g maize starch and 300 g Cr_2O_3/kg, cooked as a paste and ground; the Cr bread was added as a marker for the digestibility studies. Each diet was fed *ad lib.* for 10 d, and food consumption and weight gain were recorded. NPR was calculated by adding the weight loss of the N-free group to the weight gain of the test group and then dividing by the weight of crude protein consumed by the latter; the net digestible protein ratio (NDPR) was calculated using digested protein (crude protein consumed × ileal digestibility coefficient) rather than total protein. At the end of the experiment the rats were starved overnight and then allowed to eat for 2 h the next morning. After a further 2 h, they were killed by an intramuscular injection of 2 ml Nembutal (Abbott Laboratories Ltd, Queenborough, Kent) into the top of the hind leg, and dissected immediately. The undigested material was removed from the ileum (taken to be the last quarter of the small intestine) of each of the four rats on each dietary treatment, and bulked together.

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Faeces (from the whole period of the trial) and ileal contents were freeze-dried, ground and the faeces were analysed for Cr_2O_3, N, total and 'bound' lysine, and the lysine-isopeptides. As only limited quantities of ileal material were collected, it was analysed for Cr_2O_3, N and the isopeptides only. Using the nutrient: Cr_2O_3 concentration ratios for food and digesta, the true digestibility coefficients of N and of the different lysine components were calculated (cf. Varnish & Carpenter, 1975*b*).

A similar digestibility study was made with X 903, using more rats so as to obtain more ileal material. Ninety-six 5-week-old female rats, CFY strain, were randomly allocated to forty-eight cages so that each contained two rats. The rats in twenty-four of the cages were given the N-free diet and the remainder of the rats were given the same diet supplemented with X 903 at a level of 100 g crude protein/kg, at the expense of sucrose. The diets were fed *ad lib.* for 3 d and from each dietary treatment the faeces of twenty-four rats were collected, pooled, and stored at −20°. This was to allow duplicate samples for analysis. At the end of the third day the rats were starved overnight, and during the next morning were fed *ad lib.* for 3 h. After a further 2–2·5 h they were killed by an intramuscular injection of 0·5 ml Euthatal (May & Baker Ltd, Dagenham, Essex) and the ileal contents removed as before. From each dietary treatment the ileal contents of twenty-four rats were bulked together. Faeces and ileal contents were freeze-dried and ground for analysis.

RESULTS

Isopeptide analysis. The in vitro enzymic procedure that was used to digest test samples before isopeptide analysis did not completely hydrolyse the heated proteins. Our results (Table 1) with X 902E and BPA, both heated for 27 h at 121°, and with ileal protein from rats fed on heated X 902E, indicated that the recoveries of most amino acids after enzymic digestion were less than the amounts recovered after acid-hydrolysis, some very much less. The pattern of enzymic release from each sample was similar, although for the ileal contents the first part of the 'aminogram' was not resolved. Cystine and cysteine were not recovered at all from the enzymic digests, and this is unexplained. The corresponding values from the acid-hydrolysates can be assumed to be low due to destruction during hydrolysis. (Separate analyses with performic acid pretreatment were not carried out.) The enzymic digests also gave very low values for aspartic acid and glutamic acid. This is not explained by the amounts recovered in their isopeptides with lysine, nor as asparagine and glutamine. Although the latter compounds gave peaks that overlapped with those for threonine and serine respectively, the combined peaks in the enzymic digests were relatively small. The total recovery of lysine, i.e. as the free amino acid and in isopeptides, was approximately 50%.

In view of these findings of low recovery in the enzymic digests, the levels of isopeptides determined (see Tables 2 and 3) are referred to as 'isopeptides recovered' rather than as 'isopeptides present' in the test materials. We see no way of correcting the results for incomplete digestion by a constant factor. They have already, of course, been corrected for estimated destruction of isopeptides once released, as described previously (p. 385).

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Table 1. *Amino acid contents, determined after acid-hydrolysis or enzymic digestion, for samples of chicken muscle, X 902E, and bovine plasma albumin (BPA)*, both heated for 27 h at 121°, and of ileal contents of rats given diets containing X 902E*

(Mean values for duplicate determinations. The values obtained after acid-hydrolysis are expressed as mg/g crude protein (nitrogen × 6·25) and those after enzymic digestion as proportions of the corresponding 'acid-hydrolysate' values)

	X 902E		Ileal contents of rats given X 902E		BPA	
Digest	Acid	Enzymic	Acid	Enzymic	Acid	Enzymic
Amino acid						
Aspartic acid	95	0·06 (+0·04)†	NR	NR	98	0·22
Threonine + asparagine	43	0·72	NR	NR	45	0·93
Serine + glutamine	34	0·63	NR	NR	34	0·90
Glutamic acid	135	0·18 (+0·08)†	NR	NR	133	0·33
Proline	49	0·53	NR	NR	49	0·49
Glycine	43	0·33	NR	NR	20	0·57
Alanine	60	0·63	NR	NR	57	0·64
Valine	47	1·06	70	0·97	52	0·97
Cystine	5	0	0	0	4	0
Methionine	29	0·55	23	0·30	8	0·70
Isoleucine	49	1·09	59	1·03	21	1·00
Leucine	79	0·93	89	1·01	109	0·88
Tyrosine	37	0·93	32	1·40	49	0·94
Phenylalanine	45	0·84	44	1·11	66	0·84
Lysine	88	0·45 (+0·10)†	96	0·34 (+0·02)†	112	0·40
Histidine	22	0·68	35	0·51	35	0·46
Arginine	59	0·90	66	0·88	55	0·92
Average		0·63		—		0·66
(Average ‡)		(0·75)		(0·76)		(0·71)

NR, not resolved.

* For details of test materials, see p. 384.

† Amino acid recovered as isopeptides; no isopeptides were found in heated BPA.

‡ Average calculated from valine to arginine.

The results of analyses of samples of X 902E and X 903 (solvent-extracted and non-extracted respectively) for lysine and for isopeptides are given in Table 2. Neither lanthionine nor lysinoalanine was detected in acid-hydrolysates of these materials. The estimates of FDNB-reactive lysine by 'direct' and by 'difference' methods were essentially the same, and indicate that as the duration of heat treatment is increased more lysine becomes bound and thus inaccessible to FDNB. Aspartyl-lysine and glutamyl-lysine were not detected in enzymic digests of unheated X 902E, but they were isolated from enzymic digests of heated X 902E in similar quantities, which increased with the duration of heat treatment.

The lysine-isopeptides were also recovered from enzymic digests of X 903, but only after extraction of fat from the heated material with diethyl ether. The presence of fat appeared to inhibit the in vitro enzymic digestion. We have no values for the extent of in vitro digestion of this material after Soxhlet extraction. The lysine content of the recovered isopeptides accounted for only about 20% of those lysine units made inaccessible to FDNB by heat, in comparison with 30–50% for the heated X 902E samples.

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Table 2. *Levels of lysine and lysine-isopeptides (mg/g crude protein (nitrogen × 6·25)) recovered in acid-hydrolysates and enzymic digests of heated chicken muscle, X902E and X903*

(Mean values for duplicate determinations)

Test material*	Treatment	Acid-hydrolysates: Total lysine	Acid-hydrolysates: 'Bound' lysine	FDNB-reactive lysine: 'Difference' method	FDNB-reactive lysine: 'Direct' method	Enzymic digests: Aspartyl-lysine†	Enzymic digests: Glutamyl-lysine†	Lysine 'bound' by heat‡	Isopeptide-lysine as a proportion of lysine 'bound' by heat
X 902E	Unheated	96	3	93	89	0	0	0	—
	Heated: 2 h at 121°	94	7	87	84	0·8	0·7	4	0·5
	4 h at 121°	91	9	82	79	1·6	1·2	6	0·5
	8 h at 121°	88	13	75	73	2·0	2·0	10	0·4
	16 h at 121°	89	21	68	70	2·9	2·6	18	0·3
	27 h at 121°	89	26	63	61	4·5	4·6	23	0·4
X 903	Heated, 27 h at 116°	92	18	74	nd	1·5	0·8	15	0·2

FDNB, fluorodinitrobenzene; nd, not determined.

* For details, see p. 384.

† Calculated as lysine equivalents by taking aspartyl-lysine and glutamyl-lysine to contain 55·9 and 53·1 % lysine respectively.

‡ Calculated by subtracting 3 mg lysine/g crude protein (the 'bound' lysine value for unheated X 902E control) from the 'bound' lysine values of the heated samples.

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Table 3. *Lysine, lysine-isopeptide and lanthionine contents (mg/g crude protein (nitrogen × 6·25)) of heated proteins*

(Mean values for duplicate determinates)

Test material*	Treatment	FDNB-reactive lysine†	Aspartyl-lysine‡	Glutamyl-lysine‡	Cyst(e)ine§	Lanthionine
BPA	Unheated	122	0	0	(65)	0
	Heated:					
	8 h at 121°	96	0	0		13·2
	27 h at 121°	75	0	0		14·2
Lactalbumin	Unheated	92	0	0	(67)	0
	Heated,					
	54 h at 115°	41	5·8	5·6		12·0
Zein	Unheated	1	nd	nd	(5)	nd
	Heated,					
	54 h at 115°	0	0·6	0		5·4
Egg albumin	Unheated	63	nd	nd	(32)	nd
	Heated,					
	54 h at 115°	39	1·5	0·6		15·0
Casein	Unheated	77	nd	nd	(4)	nd
	Heated,					
	27 h at 115°	60	0·7	2·5		2·7
Blood meal, X949‖		86	1·8	1·7	(8)	0
Blood meal, X953‖		48	1·4	2·3		0

FDNB, fluorodinitrobenzene; BPA, bovine plasma albumin; nd, not determined.

* For details, see p. 384.

† FDNB-reactive lysine was determined by the 'difference' procedure. Values for lactalbumin, zein, egg albumin and casein were taken from Bjarnason & Carpenter (1970).

‡ Calculated as lysine equivalents by taking aspartyl-lysine and glutamyl-lysine to contain 55·9 and 53·1 % lysine respectively.

§ All values for cyst(e)ine are 'literature' values measured as cysteic acid after performic acid oxidation. Values for zein, egg albumin and casein are from Evans, Bandemer & Bauer (1960); for BPA from Bjarnason & Carpenter (1970); for lactalbumin from Gordon & Ziegler (1955); and for blood meal from Greenwood-Barton, Barnes, Mellon & Payne (1964).

‖ X 949 and X 953 were dried at low and high temperatures respectively; for details, see Hurrell & Carpenter (1975).

Table 3 gives the amounts of isopeptides recovered from enzymic digests of other heated proteins, together with the results of FDNB-reactive lysine and lanthionine analyses. The commercial blood meals, like heated chicken muscle, were found to contain only the lysine-isopeptides and not lanthionine. On the other hand, only lanthionine was detected in heated BPA. Both types of cross-linkage were formed in all other heated samples. Again, when the isopeptides were detected, they accounted for only about 20% of those lysine units made inaccessible to FDNB as a result of the heat treatment. Neither lysinoalanine nor ornithinoalanine was detected in any of the samples.

Animal experiments. The faeces and undigested ileal contents from rats fed on diets containing X 902E, unheated or heated for 8 or 27 h at 121°, or the N-free diet, were analysed, and the faecal and ileal digestibility values for dietary protein and for the lysine components were calculated. These values are given in Table 4 with the NPR

Table 4. *Protein quality and digestibility values for unheated and heated chicken muscle, X 902E*, fed to rats*

(Mean values for duplicate determinations. Values in parentheses represent the values for heat-treated samples as proportions of the corresponding values for the unheated control)

				Coefficient of ileal digestibility‡			Coefficient of faecal digestibility‡					
Treatment	NPR†	NDPR†	FDNB-reactive lysine ('difference method') (mg/g crude protein (nitrogen × 6·25))	N	Aspartyl-lysine	Glutamyl-lysine	N	Total lysine	'Bound' lysine	FDNB-reactive lysine ('difference' method)	Aspartyl-lysine	Glutamyl-lysine
Unheated (control)	5·5	6·2	93	0·88	nd	nd	0·98	0·99	0·93	0·99	nd	nd
Heated: 8 h at 121°	3·5 (0·64)	4·9 (0·79)	7 (0·81)	0·71 (0·81)	nd	nd	0·94	0·96	0·94	0·96	0·96	0·98
27 h at 121°	1·9 (0·35)	3·3 (0·53)	63 (0·68)	0·57 (0·65)	0·83	0·76	0·80	0·82	0·81	0·83	0·93	0·97

NPR, net protein ratio ((wt loss of N-free animals + wt gain of test animals) ÷ wt of crude protein consumed by test animals); NDPR, net digestible protein ratio (calculated as for NPR except that digested protein (crude protein consumed × ileal digestibility coefficient) rather than total protein was used); FDNB, fluorodinitrobenzene; nd, not determined.

* For details, see p. 384.

† Pooled SE of treatment means was 0·31 (2 df) for NPR and 0·45 for NDPR.

‡ For details of experimental procedures, see p. 386.

Table 5. *Digestibility coefficients for nitrogen and lysine in chicken muscle, X 903*, fed to rats*

(Mean values for duplicate determinations. Values in parentheses are the corresponding digestibility values of unheated chicken muscle, X 902E*, from the previous trial)

Digestibility technique†	N	Total lysine	'Bound' lysine	FDNB-reactive lysine ('difference' method)
Ileal	0·76 (0·88)	0·76	0·78	0·75
Faecal	0·89 (0·98)	0·91 (0·99)	0·91 (0·93)	0·91 (0·99)

FDNB, fluorodinitrobenzene.

* For details, see p. 384.

† For details of experimental procedures, see p. 386.

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and the NDPR values, and the FDNB-reactive lysine content of the chicken muscle for comparison.

With heated samples, the digestibility of the protein and the digestibility of its different lysine components were decreased, as were NPR, NDPR and FDNB-reactive lysine values. NPR decreased to a much greater extent than either the FDNB-reactive lysine values or ileal N digestibility. According to the NDPR results, the growth-promoting value of the heated protein that was digested and absorbed by the rats was greatly reduced.

Considering faecal digestibility, within each sample the values for N and for the different lysine components were similar, but those for the isopeptides were apparently slightly higher. Ileal digestibilities of N and of the isopeptides were lower than the corresponding faecal digestibilities and, for the sample heated for 27 h, the isopeptide digestibilities at the ileal level were again apparently higher than the corresponding N digestibility. Faeces from rats given X 902E heated for 27 h at 121° were also analysed for 'direct' FDNB-reactive lysine, and they were found to contain 37·0 mg FDNB-reactive lysine/g faecal protein.

As insufficient ileal material was collected from rats given X 902E for lysine analyses to be made, a similar trial was carried out on a larger scale using heated X 903. X 903 and X 902 were samples of the same chicken muscle; X 903 (cf. X 902E) was not solvent-extracted before it was heated for 27 h at 116°. Ileal and faecal digestibility values were calculated, and these are given in Table 5. The ileal digestibilities were in each instance lower than the corresponding faecal digestibilities but, within each set of results, the digestibility of N and of the different lysine components were similar. No isopeptides were recovered from ileal and faecal samples. This may have been an artefact due to the ileal and faecal contents not being solvent-extracted to remove fat before being subjected to enzymic digestion in vitro. The completeness of digestion was not checked.

DISCUSSION

Isopeptides in heated materials. The detection of glutamyl-lysine and aspartyl-lysine isopeptides in enzymic digests of heated proteins has confirmed that cross-linkages involving lysine and glutamic or aspartic acids (or their corresponding amides) can be formed in food proteins severely heated in the absence of carbohydrates. The lanthionine cross-linkage can also be formed on heating, particularly in proteins containing high levels of cystine. Most of the heated proteins we analysed contained both types of cross-linkage, although lanthionine was not formed in some low-cystine materials, such as heated chicken muscle and blood meals and, more surprisingly, the isopeptides were not recovered in the high-lysine, but also high-cystine, BPA.

Considering all the heated proteins, the isopeptides recovered accounted for 0–50% of those lysine units made inaccessible to FDNB (Tables 2, 3). Asquith & Otterburn (1971) similarly reported that the isopeptides recovered from heated wool keratin after in vitro digestion accounted for only one-third of the 'bound' lysine. It is possible that more of the 'bound' lysine is present in isopeptide form than we have succeeded in releasing by in vitro digestion and recovering for analysis. Lysine may also have

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taken part in other reactions that block its ε-amino group; no lysinoalanine was detected, but there are other possible cross-linkages involving cystine degradation products (Speakman, 1933; Philips, 1936; Bjarnason & Carpenter, 1970). It is also possible that lanthionine and other cross-linkages in the protein chain acted as a physical barrier and simply prevented the enzymic release of the isopeptides, or the penetration of FDNB, or both. These considerations apply particularly to heated BPA where, although one-third of the lysine units were made inaccessible to FDNB (Table 3), and free lysine, glutamic acid and aspartic acid were greatly reduced in enzymic digests (Table 1), no isopeptides were isolated after enzymic digestion.

Glutamic and aspartic acids were also poorly recovered from enzymic digests of heated X 902E (Table 1). Mauron (1972) suggested that these two amino acids may form imide linkages with asparagine or glutamine, thioester linkages with the thiol groups of cysteine, or ester linkages with the hydroxyl groups of threonine and serine.

Although lysinoalanine has been previously isolated from alkali-treated wool (Asquith, Booth & Skinner, 1968) and from heated wool keratin (Asquith & Otterburn, 1970), it was not detected in any of our samples. This is not surprising, since lysinoalanine would not be expected to be formed in proteins heated at acid or neutral pH (Bjarnason & Carpenter, 1970).

Animal experiments. Considering first the rat digestibility studies, it had been expected that the isopeptide linkages that had been formed in the heated chicken muscle would be more slowly digested than the rest of the protein molecule, and that they would accumulate in the ileal contents and faeces of rats given the heated material. Surprisingly the results (Table 4) seem to indicate that the isopeptides are slightly more efficiently digested by the rat than the rest of the protein molecule. This may have been an artefact due to difficulties in quantifying the amounts of isopeptides present. However, the proportion of lysine in the ileal and faecal protein was not higher than that present in the food protein, so that the digestibility of the lysine was not reduced to a greater extent than that of the other amino acids. This would be inconsistent with the isopeptide-linked lysine in the proteins being indigestible. Free glutamyl-lysine has been found to be completely available as a lysine source for rats (Mauron, 1970; Waibel & Carpenter, 1972), so that if the isopeptides were released from the protein, they would probably have been absorbed and utilized.

The apparently high rat digestibility values calculated for isopeptides result of course from the proportionally smaller amounts of these compounds isolated from the ileal and faecal protein than from the heated food protein. This could have been because some of the isopeptides were destroyed either by the bacteria, or by the additional enzymes present in the ileal and faecal samples. Attempts were made to correct for these losses by measuring the recovery of free glutamyl-lysine added at the start of the in vitro enzymic digestion procedure, but these corrections may not have been adequate for the losses of peptide-bound isopeptides. Also, when calculating the isopeptide digestibility values for the rat, it has been assumed that the extent of the in vitro enzymic digestion used to isolate these compounds was the same for both the heated protein and the ileal and faecal protein. The results in Table 1 for heated chicken muscle would seem to indicate that this is so.

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For X 903 the ileal digestibilities of N and of the different lysine components were similar (Table 5), as were ileal N digestibility and ileal digestibility of total lysine in heated X 902E. In each instance the faecal digestibility values were higher than the corresponding ileal values, indicating perhaps that bacterial fermentation had taken place in the hind gut (Nesheim & Carpenter, 1967; Valle-Riestra & Barnes, 1970; Salter & Coates, 1971), or that the 'ileal' level does not correspond to the end of enzymic digestion and absorption. Varnish & Carpenter (1975*b*) gave the same heated X 903 to chicks, and found that the ileal digestibility of all the amino acids had been reduced by similar amounts, indicating that, although severely heated protein is poorly digested, the process is not selective for particular amino acids. In other words, those amino acids involved in cross-link formation are not digested and absorbed at a slower rate than those amino acids not involved. A similar conclusion was reached by Valle-Riestra & Barnes (1970). With severely heated proteins there appears to be a general reduction in their rate of digestion, and it seems that if more time was available in contact with digestive enzymes, digestion would be much nearer completion (Buraczewski, Buraczewska & Ford, 1967).

There are certain problems in determining the 'bound' lysine content of partly digested proteins. 'Bound' lysine refers to that lysine which does not react with FDNB at either its α-amino group or its ϵ-amino group. Neither the free isopeptides nor lysine units present as N-terminal amino acids in a peptide but 'bound' at the ϵ-N position would be included in the measurement. This may explain why the 'bound' lysine digestibilities were apparently slightly higher than the digestibilities of the other lysine components (Table 4).

The detection of FDNB-reactive lysine in faecal protein suggests that lysine units with free ϵ-amino groups had passed through the digestive tract combined in indigestible peptide. Such a phenomenon would result in the measurement of FDNB-reactive lysine values over-estimating the biologically available lysine; a finding which has been reported several times in connexion with severely heated proteins (Miller, Carpenter & Milner, 1965; Ford & Salter, 1966; Boctor & Harper, 1968).

Our results indicate that 'bound' and FDNB-reactive lysine are digested and absorbed to the same extent from severely heated proteins; a finding that would be expected if the protein was digested as a whole. Previously, it had been assumed that lysine units which reacted with FDNB were nutritionally available, and as such would be completely digestible; lysine units which were inaccessible to FDNB were, in turn, thought to be unavailable to the animal because they were indigestible (Carpenter, 1957).

One other finding from these experiments was that the large decrease in nutritional value, as measured by NPR, of X 902E heated for 27 h at 121° was only partly accounted for by the reduced ileal digestibility of N or of total lysine. Donoso, Lewis, Miller & Payne (1962) reported similar findings for severely heated pork muscle.

The reasons for a reduced utilization of N are unclear. It is possible that other amino acids are more reduced in digestibility than lysine, although, as already mentioned, Varnish & Carpenter (1975*b*) reported that the ileal digestibility of all amino acids in X 903 had decreased by approximately the same extent. They also

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found that the decrease in biological availabilities of lysine, methionine and tryptophan in X 903 was similar, and was completely accounted for by their reduced ileal digestibilities. It could be that some essential amino acids, presumably other than lysine, tryptophan and methionine, are absorbed in non-metabolizable forms. Ford & Shorrock (1971) studied the possibility that some non-metabolizable small peptides may be absorbed from heat-damaged protein and excreted in the urine; they found, however, that only a very small portion (about 0·2%) of the N absorbed by rats given heat-damaged cod muscle was excreted as peptide-bound amino acids in the urine, and a further 0·6% as free amino acids. However, it might be that modified amino acids which cannot be used for protein synthesis are broken down in the body, along with excesses of other amino acids, and that their carbon skeletons are used as an energy source and their N content excreted. Racemization (Bjarnason & Carpenter, 1970; Hayase, Kato & Fujimaki, 1973) is a possible mechanism whereby an amino acid might be chemically modified, and rendered biologically unavailable.

It is still unclear which amino acid or amino acids are responsible for this reduced N utilization. The results of many experiments have indicated that in the NPR test the sulphur amino acids are the limiting factor both in good-quality and in heat-damaged fish flours (Miller, 1956; Njaa, 1961; Carpenter, Lea & Parr, 1963; Morrison & Sabry, 1963) and in unheated and heated pork protein (Donoso *et al.* 1962). As the biological availability of methionine has never been reported to decrease to a greater extent than that of lysine, it could be that the relatively large destruction of cystine, which always occurs in heated proteins, is an important factor. This is because, if all amino acids are reduced in digestibility by the same extent, then those amino acids, such as cystine, which are destroyed by heat will undergo a proportionally greater decrease in availability. Cystine, although not an essential amino acid, has a sparing action on methionine (Tarver & Schmidt, 1939). Finally, the formation of growth-reducing toxic compounds (Adrian, Frangne, Petit, Godon & Barbier, 1966) could also help to explain the low NPR and NDPR values.

It can be concluded that cross-linkages are formed in proteins during severe heat treatment, and it seems likely that they reduce the rate of protein digestion, possibly by preventing enzyme penetration, or by masking the sites of enzyme attack. However, the lysine-isopeptides themselves appeared to be at least as digestible as the rest of the protein molecule, and the reduction in ileal digestibility of N or of total lysine when the chicken muscle was severely heated accounted for only a part of the large decrease in nutritional value which resulted.

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