Mechanisms of heat damage in proteins

2.* Chemical changes in pure proteins

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1. Bovine plasma albumin (BPA) containing approximately 14% moisture, when heated for 27 h at 115° suffered an appreciable loss of cystine and a small loss of lysine; at 145° all the amino acids except glutamic acid and those with paraffin side-chains, showed considerable losses. Isoleucine also showed some loss through racemization to alloisoleucine.

2. BPA heated at 115° evolved H_2S; at 145° other sulphur compounds were released as well, all coming from the breakdown of cystine. Possible mechanisms for this are discussed.

3. Ammonia was also liberated from BPA heated at 115°. The degree of correlation of lysine binding in different proteins with ammonia liberation and amide changes has led us to suggest that the main reaction of ϵ-amino lysine groups is with amide groups of asparagine and glutamine. Reaction of ϵ-amino groups with carboxylic groups is thought to be less important.

4. Model experiments have shown that a reaction between amide groups and the ϵ-amino group of lysine in proteins can occur at practical drying temperatures.

5. Reactions of the ϵ-amino group of lysine with destruction products of cystine is also considered to be partially responsible for the lysine binding in heated proteins.

It has long been known that the Maillard reaction between sugar aldehyde groups and the free amino acid groups of proteins can explain much of the damaging effect of heat on the protein quality of dried milk. However, heat damage can also occur if meat or fish products are heated excessively and these are essentially carbohydrate-free. This too is accompanied by a loss of reactivity of the ϵ-amino groups of lysine. Any oxidizing fat present may contribute carbonyl groups for a Maillard-type reaction but high-temperature heat damage cannot be considered as being just due to reaction with other non-protein compounds present, since it will occur to the same extent with pure protein preparations (Carpenter, Morgan, Lea & Parr, 1962). The changes also occur in an atmosphere of nitrogen as well as of oxygen (Carpenter, Ellinger, Munro & Rolfe, 1957).

This study was designed to investigate the chemical changes which must occur during the heating of proteins as such, and be responsible for the nutritional damage. Because of the multiplicity of functional groups and of the many possible reactions which may occur during the heating, analytical work is difficult, and very little is known about what actually happens. Apart from amino acid destructions only the binding of lysine is well established. It has been suggested that the binding of lysine is caused by a reaction between the ϵ-amino group of lysine and carboxylic groups in the protein and that such cross-linking reduces the nutritional availability of all the essential amino acids (cf. Mecham & Olcott, 1947).

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EXPERIMENTAL

Test materials

Bovine plasma albumin (BPA) and lactalbumin were from the same source as in our first study (Bjarnason & Carpenter, 1969) and heated samples were prepared in the same way. The vacuum-dried cod fillets, Cod 23, were similar to those previously described (Miller, Carpenter & Milner, 1965). The casein was purchased as 'Casein (Hammersten)' (British Drug Houses, Poole, Dorset). The haemoglobin was crystallized beef material (Sigma Chemical Co., St Louis, Miss., USA). The egg albumen was a five times crystallized product (Nutritional Biochemicals Corp., Cleveland, Ohio, USA) and the zein was from the same source.

Analytical procedures

The moisture, total N and 'available' and 'residual' lysine in samples were determined as in our earlier experiments (Bjarnason & Carpenter, 1969).

Free ammonia. Each ampoule, containing about 1 g protein, was placed in a 1000 ml polyethylene bottle together with a heavy piece of iron; 10 ml 0·1 M-citric acid and 30 ml water were added. The bottle was secured with a screw cap and shaken vigorously to break the ampoule and mix the protein thoroughly with the liquid. After 10 min the contents were filtered and made up to 50 ml. The free ammonia was then determined in a sample of the solution according to Hamilton (1960). The unprocessed materials were analysed in the same way, except that they were placed dry in the polyethylene flask.

Total primary amide content. This was determined according to Hamilton (1960) on the dry test materials, after refluxing with 2 N-HCl for 3·5 or 7·0 h. The average of the values obtained after these two intervals was used, and the corresponding value for free ammonia was subtracted to obtain the measure of the amide content.

Total amino acids. These were determined on acid hydrolysates of the test materials prepared according to Weidner & Eggum (1966), i.e. hydrolysates were prepared, one hydrolysate in 6 N-HCl, for all amino acids except methionine and cystine, and one in 6 N-HCl after preliminary oxidation for these two amino acids. The amino acids were determined on the hydrolysates with a Beckman Amino Acid Analyser 120 B (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif., USA) by a fully automatic ion exchange column procedure described by Spackman, Stein & Moore (1958). Calculations were based on a comparison with a mixture containing all the amino acids in the concentration of 0·5 μmoles/ml except for cysteic acid; for this a special calculation technique was followed, as specified in the instruction manual supplied with the instrument.

When only the determination of total lysine was required, it was carried out on a short-column system (Roach, Sanderson & Williams, 1967).

N-terminal amino acids. These were determined with 1-fluoro-2,4-dinitrobenzene (FDNB). The methods of Fraenkel-Conrat, Harris & Levy (1955) were followed for the reaction (procedure 1), hydrolysis of the proteins in 6 N-HCl at 105° for 24 h

and extraction of the dinitrophenyl (DNP) compounds. The DNP compounds were dissolved in water-free acetic acid and the extinction of the solution was read at 435 nm, a wavelength at which dinitrophenol in acetic acid has negligible extinction. The sum of N-terminal amino acids was expressed as 'DNP-leucine equivalents', i.e. using this compound as a reference standard. A correction was made for the compound di-DNP-lysine. Portions of the acetic acid solution were evaporated in vacuo and the residues taken up in methanol and transferred quantitatively on to ready-made Kieselgel F 254 plates (Merck AG, Darmstadt, Germany) in a line at the bottom of the plates. The chromatograms were developed with benzene–acetic acid–H_2O (1:1:1 by volume, upper layer used) for direct isolation of leu (+val), phe, ala and lys (+tyr). The remaining DNP compounds could not be separated with this solvent system and were re-run for separation in n-butanol–acetic acid–H_2O (4:1:1 by volume). In this way glu, asp, ileu and ser (+thr) were isolated. The silica material containing each compound was scraped separately off the plates and the DNP compounds were eluted with dilute aqueous $NaHCO_3$. The solutions were filtered, acidified with acetic acid and evaporated. The residues were taken up in glacial acetic acid and the extinction read at 435 nm. Again, the extinction of DNP-leucine was used as a reference. For final identification, the DNP-derivatives were run in three solvent systems, the two already mentioned and chloroform–pyridine–acetic acid (40:10:1 by volume), together with authentic reference samples of each compound.

Free H_2S. The ampoules with the heated protein were placed, as for the ammonia determination, in a polyethylene bottle together with 20 ml 0·1 M-citric acid. Before breaking the ampoules, O_2-free N_2 was led through the flask for 5 min to flush out the air. Then the ampoule was broken from outside by hammering on the bottle and the nitrogen stream led for 1 h through a suspension made by mixing 10 ml 0·1 N-$Cd(OH)_2$ with 2 ml 0·1 N-NaOH. The quantitative determination of H_2S in the suspension was then carried out according to Marbach & Doty (1956). Check determinations were also made by titration with $Na_2S_2O_3$ (Association of Official Agricultural Chemists, 1965, section 31.016) and they gave essentially the same results.

Other volatile sulphur compounds. The ampoules were placed in a polyethylene tube connected with an evacuated flask. The flask had a small rubber balloon connected for pressure equilibration. After breaking the ampoules in the tube and equilibrating the pressure with N_2, a sample of gas was taken from the balloon with a syringe and injected into a gas chromatograph (Pye Ltd, Cambridge; dual flame ionization, series 104). The column was 5 ft long and contained 100–150 mesh Porapak Q. The carrying gas was argon (45 ml/min). The starting temperature was 130°, but after 60 min was increased 5°/min up to 190°. Reference materials were run both separately and mixed with the gas under study.

RESULTS

Amino acids recovered after acid hydrolysis

The destruction of amino acids at high temperatures can be measured by determining the amino acids recovered from hydrolysates of the test materials before and after they have been heat-damaged. The material used for this purpose was crystalline

BPA. It was heated at 115° for 27 h to represent the conditions of severe processing that might occur in industry and also at 145° for 27 h, i.e. under deliberately exaggerated conditions, so that small trends of losses at 115° could be confirmed. The BPA contained 13·9% H_2O in each instance. The amino acids were determined after acid hydrolysis with an amino acid analyser and the results are shown in Table 1.

Much the greatest loss occurred with cysteine, 50% at 115° and 92% at 145°. In contrast, methionine showed an apparent increase of 14 and 12% respectively but the absolute quantities are so much smaller that the differences, of approximately 0·1 g/16 g N, are probably not significant.

Table 1. *Total amino acids in bovine plasma albumin (BPA) and the effect of heating on amino acid content, liberation of ammonia and hydrogen sulphide and total weight*

		Heated (115°, 27 h)		Heated (145°, 27 h)	
	Control (g/100 g)*	g/100 g*	Change (as % of control value)	g/100 g*	Change (as % of control value)
Lysine	12·89	12·41	−4	10·91	−15
Histidine	3·70	3·68	<3	3·08	−17
Arginine	5·82	5·87	<3	5·21	−11
Aspartic acid	10·90	10·73	<3	7·38	−32
Threonine	5·69	5·50	−3	3·87	−32
Serine	3·82	3·68	−4	2·14	−44
Glutamic acid	18·31	19·06	+4	18·24	<3
Proline	4·16	4·57	+10	4·54	+9
Glycine	1·84	2·02	+10	2·53	+38
Alanine	6·12	6·17	<3	6·95	+14
Cys/2†	6·50	3·30	−50	0·51	−92
Valine	6·25	6·11	<3	6·09	<3
Methionine	0·83	0·92	+14	0·91	+12
Isoleucine	2·79	2·79	<3	2·46	−12
Allo-isoleucine	—	—	—	0·46	—
Leucine	11·98	12·06	<3	11·82	<3
Tyrosine	4·89	4·46	−9	5·15	+5
Phenylalanine	6·46	6·38	<3	6·39	<3
Tryptophan	(0·58)‡	(0·58)‡	—	(0·58)‡	—
N recovery in amino acids	15·39	15·00	—	13·39	—
Amide-N	0·70	0·70	—	0·88	—
Free NH_3-N	0·00	0·23	—	0·88	—
Total N accounted for	16·09	15·93	—	15·15	—
(N accounted for as % of Kjeldahl N in control)	(99·3)	(98·4)	—	(93·4)	—
Wt changes on heating:					
Total	—	—	−0·54	—	−3·08
Portion accounted for as NH_3	—	—	−0·27	—	−1·04
Portion accounted for as H_2S	—	—	−0·17	—	−0·47
Unexplained portion	—	—	−0·10	—	−1·57

* All values calculated per 100 g ash-free dry matter which contained 16·20 g N. In heated materials weight lost during heating was also taken into account and all values were related back to 100 g of original ash-free dry matter.

† A correction factor of 1·12 was used, bringing the determined value for control material to that found by Stein & Moore (1949).

‡ Tryptophan was not determined; the value of 0·58 (Stein & Moore, 1949) has been used for estimating the total recovery of N.

The three dibasic amino acids all show losses, amounting to 11–17% at the higher temperature but at 115° only lysine shows a loss in excess of 3%. As a general working rule, in view of the known experimental errors in the system, we are considering only differences that are at least 0·4 g/16 g N in absolute amount and representing a relative change of at least 3%.

Threonine and serine both show a high loss at 145° but not at the lower temperature. Aspartic acid does the same, but there is apparently no loss of glutamic acid. No distinction, of course, can be made between aspartic acid and asparagine, or between glutamic acid and glutamine, when analysing hydrolysates of proteins prepared by refluxing with acid as this causes hydrolysis of the amide groups.

Of the amino acids with a paraffin side-chain, there was a loss only with isoleucine. This is due to racemization, as will be explained below. However, both glycine and alanine showed significant increases, with a 10% increase in the former even at 115°.

There was the same apparent incease of 10% in the level of proline at each level of heating. Because of the greater inaccuracy of this determination, and its showing no tendency to increase at the higher temperature, no significance is attached to it.

Nearly all the N is accounted for in the material heated at 115° but, after heating at 145°, 7% has been lost even when amide-N and free NH_3 have been allowed for.

Table 4, which shows the results of another experiment to be considered later, also includes measurements of the destruction of lysine in six different protein materials each heated at 115° and 145° for 27 h. The accuracy of the total lysine values for the five materials other than BPA may be lower as they were obtained by the rapid short-column system. There is an average loss of 3% at 115° and of 19% at 145° in these six proteins. The losses vary considerably from one protein to another without any apparent relation with the amino acid composition.

Racemization

BPA heated at 145° showed a decrease in isoleucine but not in the other amino acids with paraffin side-chains (Table 1). On the amino acid chromatogram a previously unidentified peak also appeared. It was identified as allo-isoleucine (cf. Piez, 1954),

Table 2. *Contents of isoleucine, allo-isoleucine and lysine (total and bound) in vacuum-dried cod fillets, heated and unheated*

		Heat-damaged		
	Unheated	115°, 24 h	130°, 24 h	145°, 24 h
(*a*) Isoleucine (g/16 g N)	4·33	4·27	4·29	3·86
(*b*) Allo-isoleucine (g/16 g N)	0†	0·118	0·181	0·49
a+*b* (g/16 g N)	4·33	4·39	4·47	4·35
Racemization (%)	<1†	2·7	4·1	11·3
(*c*) Total lysine (g/16 g N)	8·52	—	—	—
(*d*) Bound lysine* (g/16 g N)	0·71	2·25	4·28	5·41

* Determined by the procedure of Roach *et al.* (1967).

† The hydrolysate showed a small bump at the point corresponding to allo-isoleucine on the trace but it was too small for calculation. A small degree of racemization may have occurred during the acid hydrolysis of the protein.

a racemized product of L-isoleucine, and was present in an amount equivalent, within experimental error, to the loss of isoleucine.

Examination of the amino acid chromatographs prepared from hydrolysates of cod fillets heated in various ways showed comparable results to those obtained with BPA (Table 2).

Formation of volatile compounds

The weight loss of proteins during heating gives an estimate of the volatile condensation products formed. BPA (13·9% H_2O) was placed in equal amounts in a series of ampoules and sealed. After heating some of the ampoules, they were all opened and

Table 3. *Liberation of ammonia and changes in amide content and FDNB-available lysine in five proteins as a result of heating*

(Results are given in m-equiv./16 g N unless stated otherwise)

		Analysis before heating		Changes observed as a result of heating				
		FDNB-available lysine (1)	Amide groups (2)	FDNB-available lysine (3)	Amide content (4)	Liberation of NH_3 (5)	Liberation of NH_3 as % of amide content (6)	Sum of NH_3 and amide groups (7)
Haemoglobin	Heated at 115° for 54 h	59·2	46·2	−20·8	−13·3	24·9	56	+11·6
Lactalbumin	Heated at 115° for 54 h	63·2	81·7	−35·1	−24·9	35·1	43	+10·2
Egg albumen	Heated at 115° for 54 h	43·1	86·2	−16·3	−14·5	26·0	30	+11·5
Zein	Heated at 115° for 54 h	0·7	212	−0·7	−11·8	18·3	8·5	+6·5
BPA heated at 115° for 27 h		78·6	55·4	−16·0	−6·2	16·2	29	+10·0
BPA heated at 145° for 27 h		78·6	55·4	−61·2	+7·2	61·8	112	+69·0

FDNB, 1-fluoro-2,4-dinitrobenzene; BPA, bovine plasma albumin.

the contents dried in a vacuum desiccator over P_2O_5. The difference in weight of the heated and control material was recorded as weight lost. The results are shown in Table 1. After heating and drying, some free NH_3 was still present. It was determined separately and the equivalent weight added to the weight lost.

At 115° the volatile products formed during heating seem to be mainly NH_3 and H_2S. After heating at 145° these two compounds account for only half of the total weight loss. The H_2S corresponded to 22% of the cystine destroyed at 115° and to 34% of that destroyed at 145°.

The liberation of volatile sulphur compounds other than H_2S has already been mentioned, but no quantitative determination could be made. Methyl mercaptan, dimethyl sulphide and dimethyl disulphide were detected after heating at 145°, with the use of a gas chromatograph. These compounds too must be considered as destruction products of cystine, as there had been no detectable loss of methionine.

At the higher temperature, i.e. 145°, it seems possible that additional volatile products such as CO_2 could also have been liberated. The evolution of H_2O can hardly have amounted to more than 1% of dry weight.

Changes in amide groups

The most evident chemical change in BPA heated at 115° is the liberation of NH_3. Since the destruction of amino acids, as determined after hydrolysis, is low at that

Table 4. *Contents of amide-N, cystine and lysine (total and FDNB-available) in six proteins and the effect of heat on their lysine values*

	Composition of unheated materials				Change in lysine values as a result of heating (g/16 g N)			
			Lysine (g/16 g N)		At 115° for 27 h		At 145° for 27 h	
Material	Cystine* (g/16 g N)	Amide-N* (% of total N)	FDNB-available	Total	FDNB-available	Total	FDNB-available	Total
Haemoglobin	0[1]	4·04	8·7	9·1	−1·4	−0·2	−6·2	−1·4
Casein	0·2[2]	9·12	7·7	7·8	−1·7	+0·2	−6·1	−1·6
Egg albumen	0·5[3]	6·58[3]	5·9	6·0	−1·2	−1·0	−4·5	−1·3
Cod 23	1·1[4]	10·75	8·4	8·7	−2·4	−0·6	−6·6	−2·7
Lactalbumin	6·1[5]	8·67[7]	8·2	8·0	−2·8	−0·2	−6·8	−1·6
BPA	6·3[6]	4·76	11·5	12·7	−2·3	−0·5	−9·0	−2·0

FDNB, 1-fluoro-2,4-dinitrobenzene; BPA, bovine plasma albumin.

* The cystine (not cysteine) values have been taken from the literature for each material, as have the amide-N values for egg albumen and lactalbumin.

[1]Snow (1962); [2]Wallace & Aiyar (1969); [3]Lewis, Snell, Hirschmann & Fraenkel-Conrat (1950); [4]Connell (1958); Miller, Hartley & Thomas (1965); [6]Weil & Seibles (1961); [6]Edsall (1954); [7]Gordon & Ziegler (1955).

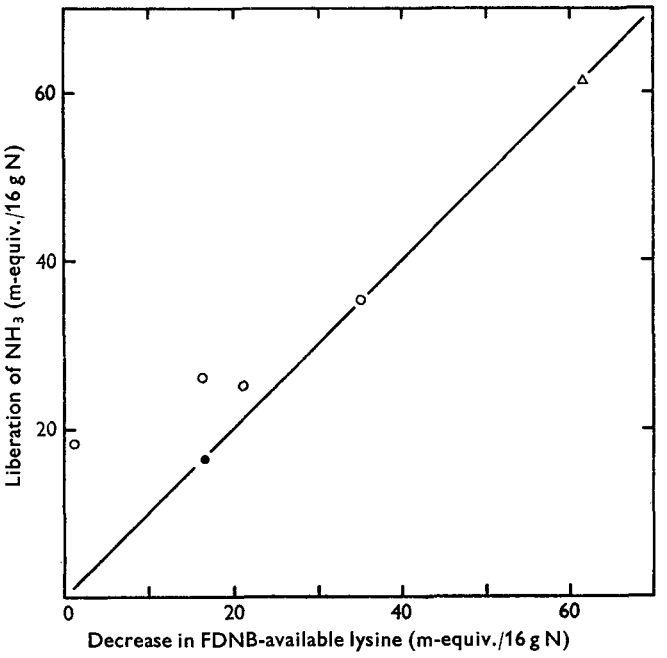


Fig. 1. The correlation of lysine binding (measured as decrease in FDNB-available lysine) and liberation of ammonia in heated proteins. ○, four materials heated at 115° for 54 h (see Table 1); ●, BPA heated at 115°, 27 h; △, BPA heated at 145°, 27 h. The straight line corresponds to a 1:1 equivalence. FDNB, 1-fluoro-2,4-dinitrobenzene; BPA, bovine plasma albumin.

temperature, it would seem that the amide groups of asparagine and glutamine must be its major source. The only alternative would be that all the N corresponding to the destroyed cystine (Table 1) was liberated as NH_3, but this seems unlikely.

To study the NH_3 liberation more closely, six different proteins were heated in sealed ampoules under N_2 after moisture equilibration over 37% H_2SO_4 in a vacuum desiccator. Free ammonia was then determined and, after drying over P_2O_5 and KOH, the total amide contents and FDNB-available lysine were also determined. The results are shown in Table 3, expressed in m-equiv./16 g N, so as to be relative to the actual number of the groups involved, and to bring out the relationship between the different changes.

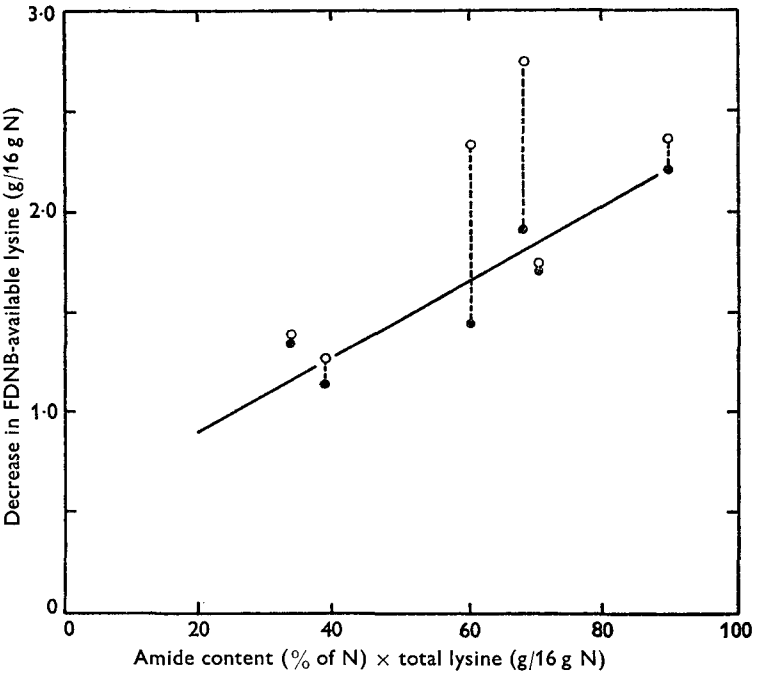


Fig. 2. The binding of lysine in proteins heated at 115° for 27 h in relation to the product of the initial concentrations of total lysine and of amide groups in the protein. ○, uncorrected values for six materials from the values set out in Table 4; ●, the same values with the change in FDNB-available lysine reduced by an amount equimolar with one-quarter of the initial cystine content of each protein.

By comparing columns 3 and 5 it can be noted that the liberation of NH_3 is in four instances approximately equivalent to the binding of lysine and in the others is higher; this is illustrated in Fig. 1. In zein there is, of course, negligible lysine available for reaction. Comparison of columns 3 and 4 indicates that the lysine binding is generally greater than the decrease in amide content.

If the major cause of lysine binding were a reaction between the ϵ-amino group of lysine and the amide groups of asparagine and glutamine, a correlation would be expected between the lysine bound during heating and the multiplication product of the initial concentrations of amide groups and total lysine respectively, so long as the

changes were relatively small. Information suitable for studying this was obtained from the results of another experiment (Table 4) in which six materials had been heated in ampoules in the usual way, and the values for the milder treatment (115°, 27 h), for which a correlation was expected, are set out in the form of a correlation diagram in Fig. 2. The possible significance of the results will be considered in the discussion.

Hydrolysis of peptide linkages during heating

A possible chemical change during the heating of proteins in the presence of water is a hydrolytic cleavage of the peptide chains. In order to study this, BPA containing 13·9% H_2O was heated at 115° and 145° for 27 h in the usual way. The N-terminal amino groups were determined with the DNP-technique, both on control and heated BPA. The results are shown in Table 5 expressed in numbers of amino groups per molecule in the intact protein, or for the corresponding weight of the heated proteins.

Table 5. *N-terminal amino acids in control and heated bovine plasma albumin (BPA) (expressed as numbers per original molecule*)*

	Intact	Heated at 115° for 27 h	Heated at 145° for 27 h
Alanine	0	0·38	1·00
Aspartic acid (+asparagine)	1·00	1·00	0·35
Glutamic acid (+glutamine)	0	0·29	0·43
Isoleucine	0	0	0·09
Leucine (+traces of valine)	0	0·32	0·66
Lysine†	0	0·21	0·25
Phenylalanine	0	0·17	0·42
Serine†	0	0·11	0·14
Total	1·00	2·48 (2·7)‡	3·34 (3·4)‡

* Assuming a molecular weight of 66 000 (Edsall, 1954; Thompson, 1954).
† Plus traces of an unknown material (see p. 321)
‡ The values in parentheses were obtained by measuring the total extinction of the mixture of DNP amino acids before chromatographic separation.

The molecular weight of BPA is *ca.* 66 000 (Thompson, 1954; Edsall, 1954) so that one molecule contains *ca.* 600 peptide links. After heating at 115° 2·7, N-terminal amino groups per mole were found. That is an increase of 1·7 which corresponds to about 0·3% of the peptide links and must be considered as very low. N-terminal histidine and arginine were not determined but their DNP-derivatives appear in the blank in the FDNB-available lysine determination, the value for which was very low. At 145° the total number of N-terminal amino groups per molecule was 3·4 which is not much higher. It is interesting to note that the N-terminal aspartic acid has decreased at the same time as all the others have increased. Glycine and cystine were not found as N-terminal amino acids; this could be due to the known sensitivity of their DNP derivatives to destruction during hydrolysis (Porter & Sanger, 1948). The unidentified traces in the lysine and serine values are probably the DNP derivatives of tyrosine and threonine respectively. Almost all the amino acids seem to appear as N-terminal after the protein has been heated in the presence of water. However, the degree of hydrolysis

is low and would correspond to an increase of only about 0·05% in dry weight due to the postulated reactions with water.

Model reactions involving amides

To investigate whether ε-amino lysine groups in proteins are acylated by heating with amides, two pure proteins were heated with acetamide and *N*-acetyl-glutamine respectively. As controls, we repeated the heat-processing in the presence of ammonium acetate and *N*-acetyl-glutamic acid monosodium salt respectively. All additives were thoroughly mixed with the proteins in a mortar and equilibrated as usual over 37% H_2SO_4 before heating. The results are shown in Table 6.

Table 6. *Effects of heat on FDNB-available lysine in haemoglobin and BPA in the presence of amides or ammonium salts*

	Total amide content before heating* (m-equiv./100 g material)	FDNB-available lysine (g/100 g protein)		Bound lysine† (g/100 g protein)
		In material	Change with heating	
Haemoglobin:				
Unheated control	46·2	8·50	—	—
Heated‡ alone	46·2	5·72	−2·78	—
Heated‡ with 14·5% ammonium acetate	39·4	4·67	−3·83	—
Heated‡ with 11·5% acetamide	236	1·52	−6·98	—
BPA:				
Unheated control	55·4	11·62	—	0·81
Heated§ alone	55·4	9·35	−2·27	2·47
Heated§ with 23·3% *N*-acetyl-L-glutamic acid monosodium salt	42·5	8·38	−3·24	3·08
Heated§ with 21·4% *N*-acetyl-L-glutamine	156·5	6·75	−4·87	4·16

FDNB, 1-fluoro-2,4-dinitrobenzene; BPA, bovine plasma albumin.

* Only primary amides measured. The ammonium salt was added in each case at a level equivalent to that of the amide, added to the same protein.

† Determined as 'unreacted lysine' after FDNB treatment and acid-hydrolysis (Roach *et al.* 1967).

‡ 115° for 54 h.

§ 115° for 27 h.

Haemoglobin heated with acetamide shows a much greater lysine binding, i.e. decrease in FDNB-available lysine, than does haemoglobin heated alone. The ratio of the initial rates of reactions must, of course, have been even higher than the final lysine binding ratio, 6·98/2·78 = 2·51, since the reaction rate decreases as the level of available lysine falls. Only 18% of the original FDNB-available lysine remains after heating with acetamide. Haemoglobin heated with ammonium acetate shows a little higher binding than when heated alone, but the effect is much less than with acetamide.

BPA heated with *N*-acetyl-L-glutamine behaves similarly. Here the final lysine binding ratio is 2·14, i.e. slightly lower than for haemoglobin, but the ratio of total amide with and without the supplement is 2·8 which is also lower than the corresponding value of 5·1 in the haemoglobin experiment. Again, with BPA the control additive *N*-acetyl-L-glutamic acid monosodium salt caused a higher binding of lysine than when BPA was heated alone. The action of the two control additives will be considered in the discussion.

From these experiments it seems clear that a reaction between the ε-amino group of lysine in proteins and primary amide groups is possible.

DISCUSSION

The losses of 50% of the cystine and 4% of the lysine from heating BPA at 115° for 27 h, as seen in Table 1, were in good agreement with the results of Miller, Hartley & Thomas (1965), who found a decrease of 60% in cystine and 6% in lysine in vacuum-dried cod heated at 116° for 27 h. Other authors (reviewed by Miller, Hartley & Thomas, 1965) report similar findings; also that the presence of carbohydrates aggravates the loss of lysine (apart from its binding) and causes losses of arginine as well, even at temperatures around 100°. Heating BPA at 145° has caused some destruction of a large number of amino acids and again similar observations have been made with vacuum-dried cod (J. Bjarnason, unpublished results).

Apparently at temperatures only a little above 100°, that is at practical drying temperatures, only cystine seems to suffer important losses in carbohydrate-free proteins. It is known that hot water releases some H_2S from wool protein and that this H_2S comes from the disulphide bridges of cystine (Schöberl, 1941). The following mechanism of a reaction between water and disulphide groups under similar conditions has been postulated (Schöberl & Eck, 1936):

$$\underset{\text{'A'}}{\text{R—CH}_2\text{—S—S—CH}_2\text{—R}} + \text{H}_2\text{O} \rightarrow \underset{\text{'A'}}{\text{R—CH}_2\text{—SH}} + \underset{\text{'B'}}{\text{HOS—CH}_2\text{—R}} \quad (1)$$

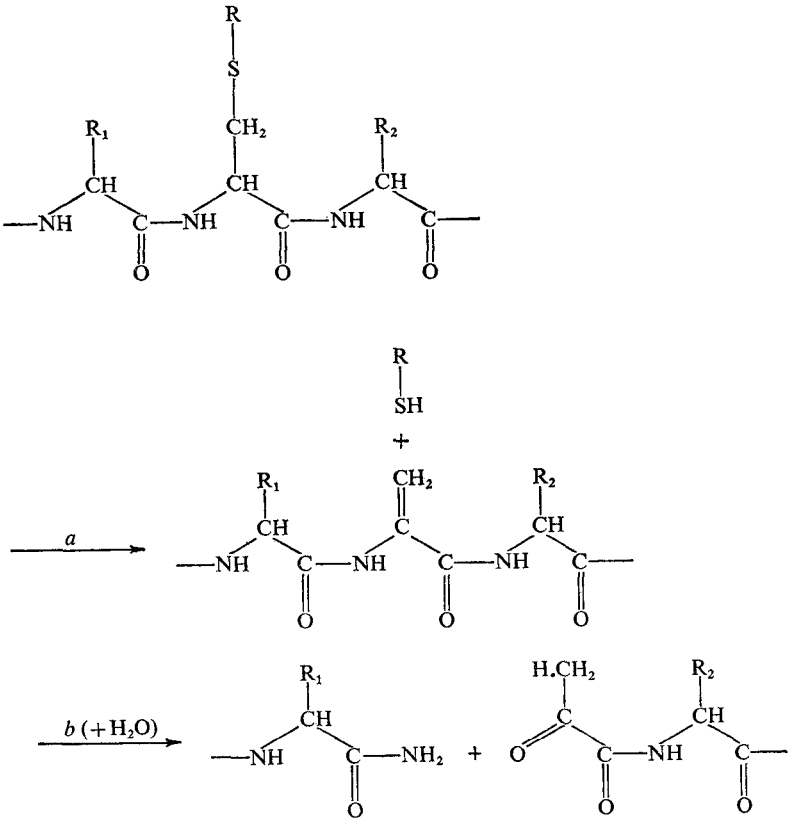
$$\text{HOS—CH}_2\text{—R} \rightarrow \text{H}_2\text{S} + \text{OCH—R} \quad (2)$$

It seems probable that such a mechanism is at least partly responsible for the loss of cystine that occurs during the heating of proteins in the absence of oxygen. After heating BPA at 115°, H_2S corresponding to about 20% of the lost cys/2 was recovered, but about 30% after heating at 145°. Similarly, Schöberl (1941) states that the recovery of H_2S after heating wool in water is not a direct measure of the disulphide cleavage as some of the H_2S will not be found and that the loss of cystine is a better indication. After heating BPA at 115° only small quantities of cystine were found after acid hydrolysis, but 51% of the original content was found after oxidation, as cysteic acid. This shows that, after heating at 115°, the cys/2 is mainly present as cysteine. The control material gave almost all of the cys/2 content as cystine. The 49% loss of the cys/2 content in this case and similar losses reported by others for a variety of materials heated above 100° (cf. Miller, Hartley & Thomas, 1965) are perhaps significant evidence of an easy destruction of one half of the cys/2 content according to equation (1).

The second stage (2) of the equation is not very clear. In some instances aldehydes have been reported as products (Schöberl & Eck, 1936) but, depending on the pH, dismutation of product B may also take place (Schöberl, 1933).

In BPA heated at 115° the sum of free and amide-bound ammonia has increased from 0·785 g to 0·925 g N/16 g N. This can only be explained by a destruction of amino acids with the transformation of α-amino-N into free or amide-bound ammonia; it cannot be explained by losses in arginine, histidine or lysine. The N must

therefore come from cystine, as there are no detectable losses in other amino acids except lysine and possibly tyrosine and it means that the peptide chain must have been ruptured. It may be that the sulphur of cystine has also been liberated to some extent through a β-elimination to form a dehydroalanine in the peptide chain, which then subsequently rearranges and easily hydrolyses to form an amide group and a pyruvic acid derivative (Patchornik, Sokolovsky & Sadeh, 1961):



Step *a* is the most common side reaction of cysteine derivatives in peptide chemistry, even at low basicities (Schröder & Lübke, 1965*b*). At neutral pH and elevated temperatures, a similar mechanism can be expected when R is —S— (a cystine derivative).

The work of Mecham & Olcott (1947) is interesting in this connexion. They found that when they heated proteins of low amide content but relatively high cystine content (i.e. hoof and egg white) in a water-free suspension in hydrocarbon the amide content increased, but that when proteins with higher amide contents and lower cystine content (casein, gluten, zein, soya-bean globulin) were heated in the same way, there was a decrease in their amide content. These results are not directly comparable with ours, since very little water was present, but they provide independent evidence that an increase in amide can take place and a further suggestion that this is to some extent related to a breakdown of cystine.

The liberation of methyl mercaptan, dimethyl sulphide and dimethyl disulphide at 145° indicates also another destruction mechanism of cystine involving the rupture of C—C bonds at that temperature. There was apparently no loss of methionine, the only other source of sulphur.

The loss of lysine recoverable from acid-hydrolysates as a result of heating BPA at 115° cannot be explained by a reaction of the ϵ-amino group with the amide groups of asparagine and glutamine. This would rather mean a protection. Table 4 shows the losses of lysine in six proteins. The two essentially cystine-free proteins, haemoglobin and casein, seem to show lower lysine losses than the others at 115° though not at 145°.

It has been demonstrated that alkaline treatment of proteins followed by acid-hydrolysis can yield *N*-ϵ-(DL-2-amino-2-carboxyethyl)-L-lysine (Bohak, 1964; Ziegler, Melchert & Lürken, 1967). It is postulated that this is the result of a break-down of cystine residues to give dehydroalanine residues and then the addition of an ϵ-NH_2 group of a lysine residue across the double bond. No peak corresponding to the lysine–alanine compound has been detected in hydrolysates of our heated samples; nor would it be expected in material that has been heated at a neutral pH.

In almost all other possible mechanisms for cystine degradation, carbonyl groups appear as products. The lysine losses during heating of proteins at 100° or above may very well be the result of a Maillard-type reaction between the ϵ-amino group and carbonyl compounds such as may result from the destruction of cystine. The apparently low degree of correlation between the lysine destruction and cystine content in the cystine-containing proteins (Table 4) may be the result of different degrees of rearrangement of Schiff's bases. Some Schiff's bases and other amino-carbonyl products are more stable than others, depending on many factors, and yield the amine component intact on acid hydrolysis (Bujard, Handwerck & Mauron, 1967).

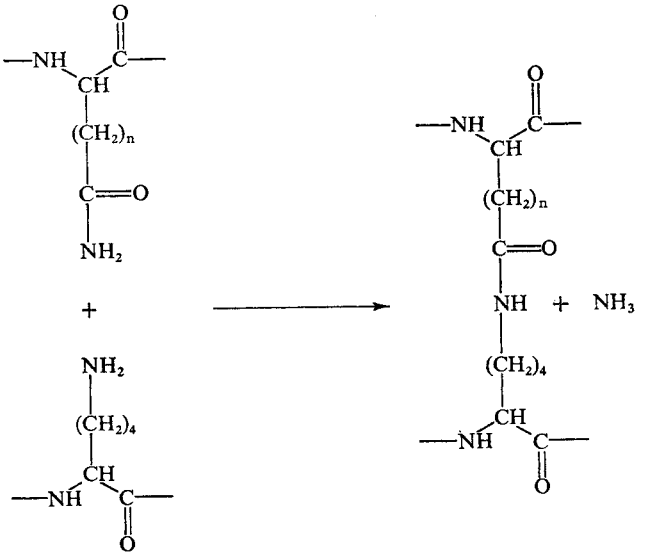
At 145° the destruction of cys/2 and lysine in BPA has increased considerably. There is a considerable destruction of histidine, arginine, serine, threonine and aspartic acid as well. The increase in free and amide-bound ammonia is 0·972 g N/16 g N. This exceeds the amount of NH_3 possibly liberated by cystine deterioration (maximal 0·699 g N/16 g N) so that other amino acids have obviously contributed. Serine and threonine are known to liberate ammonia slowly on acid hydrolysis (Smyth, Stein & Moore, 1962), probably over a β-elimination of water (classical acid-catalysed degradation of α-amino alcohols) followed by a similar rearrangement and hydrolysis to that set out in reaction *b*. Certainly the BPA had a very weak acidic pH, but an elevated temperature might have the same effect. The imidazole nucleus of histidine and the guanidyl group of arginine are potential sources of ammonia as well.

It is interesting to note that at 145° there is a small increase in glycine and a considerable increase in alanine. It is not easy to see how any other amino acid except aspartic acid could be transformed into alanine. It is known that asparagine and aspartic acid in peptide chains form α-amino-succinimide derivatives during heat treatment in boiling water (Riniker, Brunner & Schwyzer, 1962; Schröder & Lübke, 1965*a*). Such products may possibly decarboxylate to some extent to give alanine; the loss in aspartic acid is in line with this. It is interesting that irradiation has also been found commonly to increase the alanine content of proteins (Roubal & Tappel, 1966).

The total N recovery was almost 100% both for intact BPA and for BPA heated at 115° (Table 1). After heating at 145°, 6·5% of the N remained unaccounted for; this will be included in other nitrogenous compounds than amino acids or free and amide-bound ammonia, as a result of more complex deterioration.

The changes in amino acid composition during heating of proteins, which have already been discussed, are not of much nutritional significance unless the destruction of cystine were to make the sulphur amino acids limiting. Normally, other changes in the proteins affecting availability reduce the nutritive value much more, overriding the relatively small changes in amino acid composition (cf. Miller, Hartley & Thomas, 1965). As the main nutritional damage is not detected by amino acid analyses on acid-hydrolysates, it is obvious that the changes responsible are largely reversed on acid hydrolysis, that is to say, they are a result of condensation reactions. The binding of lysine during the heating of carbohydrate-free proteins, which has been known for some time (Carpenter *et al.* 1957, 1962; Lea, Parr & Carpenter, 1960) must be of this nature, as chemically bound lysine can be liberated on hydrolysis, and is measured as residual lysine in the procedure of Roach *et al.* (1967).

It has sometimes been suggested that the binding of lysine in carbohydrate-free proteins during heating is due to the formation of unnatural 'amide' bonds between the ϵ-amino group of lysine and carboxylic groups in the proteins. Certainly these would be reactions that could not be detected by total amino acid analyses. Our results suggest the formation of the same products but by reaction between the ϵ-amino group of lysine and the amide groups of asparagine ($n = 1$) or glutamine ($n = 2$), with the reacting units either in the same peptide chain or in neighbouring ones.



The following points, both from our results and from the literature are in favour of this.

(1) As seen in Table 1, ammonia is the main volatile product formed during the heating of BPA at 115°. There is reason to believe that the liberation of water as a

result of condensation between ε-amino groups of lysine and carboxylic groups is low if any, unless there is some unexpected chemical uptake of water, as the recovery of weight loss in NH_3 and H_2S is about 82% (Table 2).

A water uptake according to reaction *b* above would only amount to about 0·1% of the dry weight and, against this, the carbonyl compounds formed could lose the added water in condensation reactions. The water uptake as a result of hydrolytic cleavage of peptide bonds is extremely low, as has already been discussed.

(2) Liberation of NH_3 in five proteins heated at 115° for 54 h is higher than or equivalent to the lysine binding (Table 3). The liberation is not a direct function of the amide content alone, as is seen in column 7, which indicates that it is the result of some interaction though, obviously, small amounts of NH_3 may also be liberated by hydrolysis of the amide groups.

Zein which has far the highest amide content does show a small liberation of NH_3, even though it has almost negligible lysine content. This represents a deviation from the general correlation between the lysine binding and the liberation of NH_3 (Fig. 1). Four of the proteins show lower amide decreases than lysine binding. Here again zein is an exception, but the relative change in the amide content of this material is low.

The decrease in the amide content as a result of some specific reaction is complicated by the fact that, mainly at higher temperatures, there is another factor causing the formation of amide groups. This is seen from BPA heated at 145° and from the results of Mecham & Olcott (1947). Column 7 in Table 3 gives an idea how much this could amount to at 115°.

(3) Table 4 shows that the lysine binding in six proteins seems to be dependent on the amide content of the protein as well as on the lysine content. This suggests that a bimolecular reaction is involved and one would expect the rate of reaction to be roughly proportional to the product of the concentrations of each reactant. A correlation diagram showing the available information has been plotted in Fig. 2.

If, as has been argued above, the destruction products of cystine also play a part in the binding of lysine through their carbonyl groups, one would have to allow for this before estimating the extent of the reaction between amide groups and lysine. In Fig. 2 a deduction has been made for this on the arbitrary assumption that one-half of the cystine has been destroyed and that one-half again of the destroyed molecules have yielded a carbonyl group which has combined with lysine. Since the molecular weights of lysine and cystine are in the ratio 146 : 240, the change in FDNB-lysine (g/16 g N) has been reduced by '0·14 × cystine content (g/16 g N)'. It is seen that these adjusted values do show a closer correlation with the product of 'initial amide content' and 'total lysine content'.

(4) Model experiments on haemoglobin and BPA, heated with acetamide and *N*-acetyl-glutamide respectively, showed that a reaction between amide groups and the ε-amino group of lysine in proteins can occur under the conditions of our heating experiments.

The same proteins heated with ammonium acetate or *N*-acetyl-glutamic acid monosodium salt, also showed a higher lysine binding than the proteins heated alone, but the effect was much less than when the amides were added. Perhaps these control

materials were poorly chosen as ammonium acetate contributes ammonia, which could transform cyclic aspartic and glutamic acid residues into amides and so indirectly increase the amide content of the protein. *N*-acetyl-glutamic acid monosodium salt also contains a secondary amide group, which could possibly acetylate the ϵ-amino group of lysine to some extent.

Reaction between carboxylic groups and the ϵ-amino group of lysine is not very likely on thermodynamic grounds. High temperatures and a removal of water are needed to bring about an appreciable condensation between these groups.

(5) Ford & Salter (1966) found that aspartic and glutamic acids in cod fillet heated at 135° were poorly released by enzymic digestion as compared with amino acids other than lysine and cystine. Erbersdobler, Dümmer & Zucker (1969) studied the absorption of amino acids from heated casein in the portal blood of growing rats. They found glutamine and asparagine to be the acids most reduced after lysine in availability in protein that had been heated at 105°. Our results suggest that simple hydrolysis during heating of the amide groups of these amino acids is low. Thus, the findings of Erbersdobler *et al.* (1969) seem to indicate a specific reaction involving these two amino acids.

(6) ϵ-(γ-Glutamyl)-lysine has been isolated from enzymatic hydrolysates of cross-linked human fibrins (Pisano, Finlayson & Peyton, 1968; Matačić & Loewy, 1968) but was not found in the intact fibrin. This cross-linking reaction was catalysed by the enzyme plasma transglutaminase, i.e. it involved glutamine rather than glutamic acid. It is rather unlikely that this enzyme enables the reaction to proceed through an energy source carried by the enzyme. This emphasizes that a reaction between the amide groups and the ϵ-amino group of lysine in proteins is at a thermodynamic advantage, even at low temperatures.

The nutritional significance of such cross-linkages will be discussed in a further paper.

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