# Mechanisms of heat damage in proteins

## 1. Models with acylated lysine units*

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1. ε-*N*-Acetyl-L-lysine showed a growth-promoting value for the young rat receiving a lysine-deficient diet approximately half that of the equivalent quantity of L-lysine; ε-*N*-propionyl-L-lysine showed negligible activity.

2. Considerable quantities of lysine were recovered from acid-hydrolysis of the urine of rats receiving these two compounds. Qualitative chromatography suggested that the compounds themselves were appearing in the urine.

3. Similar results were obtained from giving proteins that had the ε-$NH_2$ groups of their lysine units either acetylated or propionylated.

4. Giving a pure protein in which the nutritional availability of the lysine had been reduced by heat treatment resulted in greatly increased faecal lysine but little urinary lysine.

The fall in nutritive value of the proteins in foods containing reducing sugars, which can occur as a result of relatively mild conditions of processing or storage, seems to be fully explained by Maillard reactions. These occur between the carbonyl groups of the sugars and the free amino groups of the proteins to yield enzyme-resistant linkages that make a portion of the amino acids, particularly lysine, nutritionally unavailable. Similar losses in nutritive value can occur in foods that do not contain sugars (or other molecules containing free carbonyl groups), though only with more severe processing conditions. It has often been hypothesized that these changes are due to the formation of unnatural 'amide' bonds between the ε-$NH_2$ group of lysine (or other free amino groups) and carboxyl groups in the proteins. We will be concerned in this first paper with the question of whether or not such linkages can be expected to be resistant to digestive enzymes and in a further paper with the evidence for and against the formation of such compounds when proteins are heated.

The only compound of this general type to have been investigated so far appears to be ε-*N*-acetyl-L-lysine which was reported by Neuberger & Sanger (1943) to promote the growth of lysine-deficient rats, both as an oral supplement and when given by intramuscular injection. However, it is now known that mammalian kidneys contain ε-lysine acylase which hydrolyses ε-*N*-formyl and acetyl lysines but is almost inactive with larger acyl groups (Paik & Benoiton, 1963; Leclerc & Benoiton, 1968*a*).

We have investigated the nutritional effects of acylation of the ε-$NH_2$ group of lysine as such, and of lysine units within whole protein chains. We have also attached propionyl groups as well as formyl and acetyl groups.

* Some of these results have been communicated in a preliminary form (Bjarnason & Carpenter, 1969).

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## EXPERIMENTAL

### *Test materials*

*L-Lysine hydrochloride.* The lysine used for the standard was natural L-lysine mono-hydrochloride (chromatographically pure grade; Mann Research Laboratories, New York).

*ε-N-Acetyl-L-lysine.* This material was synthesized from the same sample of L-lysine hydrochloride according to Neuberger & Sanger (1943). It was crystallized to a constant m.p. of 232–233°. Elementary analysis gave the following results (with the expected values in parentheses): C 50·85 (51·04), H 8·73 (8·56) and N 14·61 (14·88).

*ε-N-Propionyl-L-lysine.* This was prepared by essentially the procedure used for acetyl lysine with propionic anhydride replacing acetic anhydride. The yield was low through losses during the crystallization needed for complete separation from unreacted lysine. The compound (m.p. 237–238°) was chromatographically pure by the ninhydrin test. The elementary analysis was C 53·48 (53·44), H 8·91 (8·96) and N 14·03 (13·85). (The synthesis of this material by a slightly different method has since been described by Leclerc & Benoiton, 1968*b*).

*Bovine plasma albumin (Cohn fraction V).* This material (BPA) was a commercial sample (Koch-Light Laboratories, Colnbrook, Bucks). It contained 1·63% ash, 4·13% moisture and 15·27% N.

*Acetyl-BPA.* To prepare this material 20 g BPA were dissolved in 200 ml $H_2O$ and 200 ml saturated sodium acetate solution were added. To the ice-cooled solution were added 35 ml acetic anhydride over 1 h with constant stirring. The solution was stirred for a 2nd hour with the mixture rising to room temperature and 7 l. ethanol were then added. After standing overnight, the precipitate was separated by filtration, washed with ethanol and completely redissolved in 200 ml $H_2O$. With the addition of 2 l. ethanol a voluminous precipitate formed; this was centrifuged, washed with ethanol and spread on filter paper to dry. Finally it was kept in a desiccator over $P_2O_5$ and NaOH. The acetylation procedure was that used by Fraenkel-Conrat, Bean & Lineweaver (1949) and found to be specific for ε-amino groups of lysine (Olcott & Fraenkel-Conrat, 1947).

*Lactalbumin X.844.* This was a commercial sample (Nutritional Biochemicals Corporation, Cleveland, Ohio) of 'purified' grade. It contained 12·73% N and 5·0% moisture.

*Formyl lactalbumin.* To prepare the material 60 g lactalbumin were suspended in 250 ml formamide and 4·7 g acetic anhydride (dissolved in 50 ml formamide) were added over 15 min with stirring, at room temperature. After 6 h of stirring, 1·5 l. ethanol and 0·5 l. diethyl ether were added. A precipitate fell to the bottom and was separated by decanting and three washings with ethanol. It was centrifuged and rewashed, dried and kept over $P_2O_5$ and NaOH. (This followed the method for specific formylation of lysine developed by Vratsanos (1960).) It contained 14·47% N, some of the N being due possibly to a small retention of formamide.

*Propionyl lactalbumin. I.* Following the procedure of Tazawa (1943), 60 g

lactalbumin were suspended in 80 ml propionic anhydride and the mixture was kept for 3 days at room temperature with occasional shaking. It was then diluted with diethyl ether; the solids were separated by decanting and washed with ether. Because this procedure was expected to cause some o-acylation of phenolic and hydroxylic groups, the procedure of Hendrix & Paquin (1938) was used to hydrolyse these groups. The preparation was suspended in 500 ml $H_2O$ and sufficient 0·1 N-NaOH was added to obtain a pH of 9. For the next 4 h further NaOH was added to maintain this pH. No further addition was needed for the last hour. The suspension was then neutralized with acetic acid and the precipitate separated and washed. The dry sample contained 14·33 % N. (As will be seen below, this procedure resulted in only partial propionylation of the lysine residues.)

*Propionyl lactalbumin. II.* We followed the procedure used for the acetylation of BPA with sodium propionate in place of sodium acetate and propionic anhydride in place of acetic anhydride. The product contained 13·7 % N on a dry-matter basis.

### *Controlled heat-processing*

Materials were adjusted to a standard vapour pressure over 37 % (w/w) $H_2SO_4$ in an evacuated vessel. Samples were then sealed in ampoules and heated as described by Carpenter, Morgan, Lea & Parr (1962).

### *Analytical methods*

*Moisture.* This was determined by weight loss when the material was kept over $P_2O_5$ and NaOH for at least 24 h in an evacuated desiccator.

*Nitrogen.* N was determined by the Kjeldahl method using a macro-digestion procedure (Association of Official Agricultural Chemists, 1965, section 2.044), followed by semi-micro distillation of the ammonia produced into 1 % (w/w) boric acid containing a mixed indicator for titration with 1/70 N-HCl (Ma & Zuazaga, 1942).

*Total lysine.* This was determined on acid-hydrolysates of test materials in the way described by Roach, Sanderson & Williams (1967), using short-column chromatography with a Technicon Autoanalyzer (Technicon Ltd, Chertsey, Surrey).

*FDNB-available lysine.* The procedure was that of Carpenter (1960) in which test materials are treated with fluorodinitrobenzene (FDNB), then hydrolysed with acid and the resulting ε-dinitrophenyl lysine (DNP-lysine) is taken as a measure of the original 'available lysine'.

*Residual lysine.* Residual lysine was determined in the same hydrolysates (i.e. after initial treatment with FDNB) as the lysine which was present as such (i.e. which had not formed the DNP derivative), following Roach *et al.* (1967). The value for the residual lysine was then subtracted from that for the total lysine to give an alternative measure of available lysine 'by difference', as first suggested by Rao, Carter & Frampton (1963).

*Thin-layer chromatography.* This was carried out with ready-made Kieselgel F 254 plates (Merck AG, Darmstadt, Germany). The tests were run with either n-butanol: acetic acid:$H_2O$ (4:1:1) or n-propanol:concentrated $NH_4OH$:$H_2O$ (6:3:1). The

spots were developed with ninhydrin. Using pure compounds, the following $R_f$ values were obtained:

| | Butanol–acetic acid | Propanol $NH_3$ |
|---|---|---|
| Lysine hydrochloride | 0·03 | 0·38 |
| Acetyl-lysine | 0·18 | 0·66 |
| Propionyl-lysine | 0·27 | 0·61 |

### *Growth assay for lysine*

The procedure followed that described, in abstract form only, by Carpenter (1957). The basal diet consisted of wheat gluten 6·5, dried skim milk 6·0, zein 10·0, sesame-seed meal 9·0, L-tryptophan 0·1, DL-methionine 0·3, L-histidine 0·25, L-threonine 0·3, L-isoleucine 0·3, L-valine 0·4, arachis oil 5·0, potato starch 10·0, a mineral pre-mix 4·0, vitamin pre-mix (Chapman, Castillo & Campbell, 1959) 1·0 and maize starch to 100. The mineral mix contributed the following salts (as % of the whole diet): calcium citrate 1·23, $Ca(H_2PO_4)_2.H_2O$ 0·45, $K_2HPO_4$ 0·87, KCl 0·50, NaCl 0·31, $CaCO_3$ 0·27, 3 $MgCO_3.Mg(OH)_2.3H_2O$ 0·14, $MgSO_4$ 0·15, $MnSO_4.5H_2O$ 0·02 and the following additional salts (as ppm of the diet): $ZnCO_3$ 40, ferric citrate 650, $CuSO_4.5H_2O$ 45, NaF 6, $K.Al(SO_4)_2.12H_2O$ 4 and KI 2.

The experimental diets were prepared by adding supplements, as indicated in Table 2, at the expense of maize starch.

Gnotobiotic, male CFE rats (Lane-Petter, Lane-Petter & Bowtell, 1968) were purchased at a weight of 40–45 g (corresponding to 18–20 days of age) and transferred to our experimental room, which was maintained at 29–31°. In Expts 1 and 2 they were kept for 2 and 4 days respectively on the basal diet alone and then divided at random into individual cages where they received the dry experimental diets *ad lib.* for a further 8 days. Their weight gains and food consumption over this period were recorded. There were four rats per treatment.

For each experiment a straight line was fitted to the response plot of 'g gain/g food eaten' against level of supplementary lysine, and an estimate of the potency of each supplement was read off according to the response obtained with it.

### *Metabolic studies*

For these studies both the rats and the diets were similar to those used for the growth assays. The test diets were, however, all constructed so that the supplement contributed an estimated 0·20–0·27% of total lysine above that in the basal diet alone: a further addition of L-lysine was made to bring the contribution of 'available lysine' up to an estimated 0·20% in Expt 4 and 0·25% in Expt 5, where there was reason to think that some of the lysine in the supplement was in an unavailable form. In this way it was hoped to have all the test rats growing at a similar rate. At randomization two rats were allotted to each test diet and kept individually in metabolism cages allowing separate collection of urine and faeces. Each was transferred to a second cage for one feeding period of an hour in the morning and another in the evening. After each period the rats were put together for a few minutes romping in which any dust from the food appeared to be brushed off their fur.

Urine and faeces were collected for 4 or 5 days after a preliminary 3 days during

which the rats received the experimental diets. Portions of the urine were analysed for free lysine by direct chromatography using the Technicon Autoanalyzer, and further portions were analysed for 'total lysine' after acid-hydrolysis as described above. Faecal samples were analysed for total lysine only. In calculating the results, the determined urine values were multiplied by 1·1 to correct for the proportion of time that the rats spent out of the metabolic cages. In Expt 4 the same correction was applied for the faecal recoveries, but in Expt 5 the faeces shed during the feeding periods were added to those collected during the periods in the metabolism cages.

## RESULTS

### *Chemical analyses*

The results are summarized in Table 1. It is seen that the FDNB-available values obtained by the direct procedure were always lower than those obtained by difference, though the ranking of the samples by the two procedures was almost identical.

Table 1. *Analytical results on the test protein preparations used* (% *of dry matter*)

| | | Lysine | | | |
|---|---|---|---|---|---|
| | | | Residual (unreacted with FDNB) | FDNB-available | |
| | Kjeldahl nitrogen | Total | | By difference | Direct |
| BPA | | | | | |
| Unheated | 15·9 | 12·7 | 0·8 | 11·9 | 11·6 |
| Heated to 145° for 27 h | 15·8 | 10·7 | 7·9 | 2·8 | 2·6 |
| Acetyl-BPA | 15·1 | 11·8 | 9·0 | 2·8 | 1·8 |
| Lactalbumin | | | | | |
| Unheated | 13·3 | 9·0 | 0 | 9·0 | 7·2 |
| Heated to 115° for 54 h | ND | 8·8 | 3·1 | 5·7 | 5·1 |
| Formyl lactalbumin | | | | | |
| Unheated | 14·5 | 10·6 | 6·3 | 4·3 | 3·0 |
| Heated to 115° for 54 h | ND | 9·6 | 7·2 | 2·4 | 2·0 |
| Propionyl lactalbumin I | 14·3 | 9·5 | 3·7 | 5·8 | 5·2 |
| Propionyl lactalbumin II | 13·7 | 8·8 | 8·1 | 0·7 | 0·2 |

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

Both the pure untreated BPA and lactalbumin gave direct FDNB-available values that were lower than the corresponding total lysine values by 10–20%. The formyl and acetyl preparations appeared to have 75 and 82% respectively of their $\epsilon$-lysine groups in a form that would not give a direct FDNB reaction. Propionyl lactalbumen I had less than half its lysine groups made unavailable to FDNB, but in preparation II they appear to have been almost completely bound.

The heat treatments given to the test materials resulted in significant falls in the levels of FDNB-available lysine, but smaller changes in total lysine values.

### *Growth experiments*

The treatment means set out in Table 2 have been adjusted for differences in the initial weights of the rats in the experiment. This adjustment was made because an

analysis of covariance showed that initial weight had had a significant effect on the food conversion efficiency. The rats in both Expt 1 and Expt 2 showed a satisfactory linear response to the different levels of supplementary lysine and the variability between individuals was reasonably low.

Table 2. *Response of rats receiving a lysine-deficient diet to either lysine or compounds yielding lysine on acid-hydrolysis*

| Description | Test supplement: Level of inclusion (g dry matter/100 g diet) | Test supplement: Total lysine contributed (mg/100 g diet) | Wt gain of rats (g)/g food eaten: Expt 1 | Wt gain of rats (g)/g food eaten: Expt 2 | Estimated % activity of the lysine in supplement |
|---|---|---|---|---|---|
| None (negative control) | — | — | 0·160* a† | 0·095 a | — |
| **Amino acid** | | | | | |
| L-Lysine hydrochloride | 0·084 | 67 | 0·227 bcd | — | (100) |
| | 0·125 | 100 | — | 0·207 bc | |
| | 0·167 | 133 | 0·288 de | — | |
| | 0·250 | 200 | 0·378 f | 0·326 de | |
| | 0·375 | 300 | — | 0·447 f | |
| ε-Acetyl-L-lysine | 0·257 | 200 | 0·260 cd | — | 50 |
| ε-Propionyl-L-lysine | 0·277 | 200 | 0·158 ab | — | 0 |
| **Protein** | | | | | |
| BPA | 1·54 | 195 | 0·331 ef | — | 85 |
| Acetyl-BPA | 1·55 | 183 | 0·278 cde | — | 67 |
| Lactalbumin | 2·44 | 220 | — | 0·408 ef | 122 |
| Formyl lactalbumin | 2·05 | 217 | — | 0·288 cd | 77 |
| Propionyl lactalbumin I (40% propionylation) | 2·05 | 200 | — | 0·327 de | 107 |
| Propionyl lactalbumin II (95% propionylation) | 2·05 | 180 | — | 0·183 b | 43 |
| **Heated protein** | | | | | |
| 145°, 27 h | | | | | |
| BPA | 1·54 | (195)‡ | 0·186 ab | — | 13‡ |
| Acetyl-BPA | 1·55 | (183)‡ | 0·217 abc | — | 32‡ |
| 115°, 54 h | | | | | |
| Lactalbumin | 2·26 | (203)‡ | — | 0·215 bc | 52‡ |
| Formyl lactalbumin | 2·05 | (215)‡ | — | 0·207 bc | 46‡ |
| Standard error of treatment means | | | (0·021) | (0·028) | |

BPA, bovine plasma albumin.

* This treatment was replicated three times and the mean had a standard error of ±0·012.

† Within each column, values are significantly ($P < 0·05$) different if they do not share a common letter (Duncan's test).

‡ The total values taken here for the heated proteins were the amounts of lysine found in the corresponding samples before heating.

Because some of our test materials were in short supply it was not possible to use more than one level of each. This means that, although the responses were calculated from the standard response in terms of equivalent lysine activity, no valid statistical test could be made of the precision of these estimates. It is clear, however, that the rats did respond to the supplement of acetyl-lysine (but not to the same extent as to lysine itself) whereas they showed no response to propionyl-lysine at the equivalent dose.

In each experiment, acylated proteins gave a lower response than the equivalent supplement of unmodified protein, though with acetylated BPA the difference did not reach statistical significance. The smallest response was obtained with the almost completely propionylated lactalbumin II, but it was still significant. From the results with heat-processed samples there was a suggestion that formylated and acetylated materials were less sensitive than unmodified proteins to further damage. Thus, heating BPA alone at 145° for 27 h resulted in an apparent fall in estimated lysine activity of 72 units (from 85% to 13%) whereas the same treatment of acetyl-BPA resulted in a fall of only 35 units (67% to 32%).

The lactalbumin as purchased gave a response greater than would have been expected even on the assumption that all its lysine was biologically available. Although the difference did not quite reach statistical significance it is in line with other observations. We hope to investigate this further.

### *Metabolism of acylated lysine*

The results of the two metabolism trials are set out in full in Table 3. It can be seen that only a low level of free lysine was found in the urine with any of the test diets. The unmodified protein material BPA also gave the same low level of bound lysine as was found with the equivalent dose of pure lysine (i.e. the control treatment).

The acylated materials all gave high urinary levels of bound lysine. With both propionyl-lysine and the propionyl lactalbumin II these excretions were equivalent to over 40% of the lysine ingested from these supplements. Thin-layer chromatography of these urines with the propanol–$NH_3$ mixture showed the appearance of extra spots moving with propionyl-lysine and no others, but no attempt was made to determine the quantity of lysine in these spots.

Although little bound lysine was recovered from the urine of rats receiving the severely heated BPA, quantities equivalent to nearly two-thirds of that ingested were recovered in the faeces. The apparent faecal excretion of lysine from the other derivatives was smaller. The negative value, of the same order, obtained with propionyl lactalbumin may indicate experimental variability; there seems to be no point in attempting an explanation for such a value until the observation is confirmed.

## DISCUSSION

The experiments have confirmed the observation of Neuberger & Sanger (1943) that ε-*N*-acetyl-L-lysine has growth-promoting activity for lysine-deficient rats. However, it has shown only about one-half the activity (mole/mole) of lysine itself and rats receiving it excreted in their urine a significant quantity of material giving lysine on hydrolysis. This was probably unmetabolized acetyl-lysine.

In contrast, ε-*N*-propionyl-L-lysine had no detectable growth activity for the rat. It is interesting that, as shown in Table 4, the propionyl derivative also differed from the acetyl one in not being hydrolysed by rat kidney ε-lysine acylase (Leclerc & Benoiton, 1968*b*).

Table 3. *Urinary and faecal excretion of total lysine (determined after acid hydrolysis) by individual rats receiving different test supplements*

| Test supplement: Description | Level in diet (g dry matter/100 g diet) | Total lysine contributed to diet (mg/100 g) | Lysine added to the diet as lysine (mg/100 g) | Weight of rat (g): Initial | Weight of rat (g): Change during test* | Food eaten over period* (g) | Faecal lysine (mg/100 g diet eaten): Total | Faecal lysine (mg/100 g diet eaten): Difference from control rats | Urinary lysine (mg/100 g diet eaten): Free lysine | Urinary lysine (mg/100 g diet eaten): Total | Urinary lysine (mg/100 g diet eaten): Difference from control rats | Lysine from test supplements excreted (%): In faeces | Lysine from test supplements excreted (%): In urine |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Expt 4 | | | | | | | |
| Control | — | — | 133 | 49 | + 6 | 29·0 | 83 } 89 | — | 0·9 | 5·9 } 5 | — | — | — |
| | | | | 52 | + 7 | 33·1 | 95 } | | 1·2 | 4·3 } | | | |
| Acetyl-lysine | 0·305 | 237 | — | 56 | + 6 | 40·9 | 128 | +39 | 0·7 | 44 | +39 | | |
| | | | | 52 | + 7 | 31·7 | 133 | +44 | 0·7 | 49 | +44 | 18 | 18 |
| Propionyl-lysine | 0·327 | 237 | 133 | 53 | + 9 | 32·2 | 118 | +29 | 1·1 | 98 | +93 | | |
| | | | | 48 | + 8 | 31·8 | 151 | +62 | 1·9 | 104 | +99 | 19 | 41 |
| BPA | | | | | | | | | | | | | |
| Unheated | 1·04 | 132 | — | 52 | + 5 | 28·0 | 126 | +37 | 0·9 | 6·6 | + 2 | | |
| | | | | 49 | + 6 | 37·8 | 88 | − 1 | 1·0 | 5·6 | + 1 | 14 | 1 |
| Heated to 145° | 1·04 | 111 | 133 | 50 | + 9 | 33·8 | 161 | +72 | 0·7 | 9·1 | + 4 | | |
| for 27 h | | | | 52 | + 8 | 33·0 | 164 | +75 | 0·6 | 8·7 | + 4 | 66 | 4 |
| | | | | | | Expt 5 | | | | | | | |
| Control | — | — | 250 | 59 | + 9 | 24·9 | 141 } 143 | — | 1·5 | 4·5 } 5 | — | — | — |
| | | | | 58 | + 8 | 20·7 | 145 } | | 1·8 | 5·3 } | | | |
| Propionyl lact- | 2·59 | 228 | 250 | 49 | +11 | 19·8 | 123 | −20 | 2·8 | 102 | +97 | | |
| albumin II | | | | 57 | + 6 | 18·3 | 91 | −52 | 3·2 | 93 | +88 | (−16) | 41 |

BPA, bovine plasma albumin.

* In Expt 4 the experimental period was 5 days and in Expt 5 4 days.

The results of feeding propionyl lactalbumin II, in which almost all the ε-lysine groups were acylated, suggest two things. First, the growth response indicates that there must have been significant hydrolysis of the acyl groups—perhaps by gut enzymes, since the protein would remain there longer than the dietary supplements of propionyl-lysine which can apparently be absorbed as such. Secondly, the recovery of bound lysine, probably propionyl-lysine itself, from the urine indicates that the α-peptide linkages of lysine units may be hydrolysed in the intestine even though the ε-$NH_2$ group is blocked. Trypsin does not hydrolyse peptides at the carboxyl group of lysine units if the ε-$NH_2$ group of the lysine is blocked (cf. review by Hill, 1965).

Table 4. *A comparison of the results from the rat growth and metabolism experiments, using acylated materials, with the in vitro enzymic results of Leclerc & Benoiton* (1968*b*)

| | | | Estimated partition of the lysine from the test materials in the rat experiments | | | |
|---|---|---|---|---|---|---|
| | % of lysine ε-$NH_2$ groups in sample unreacted* | Relative activity of substrates for rat kidney ε-lysine acylase (Leclerc & Benoiton, 1968*b*) | % available for growth | % excreted | | Sum of c+d+e |
| | | | | Faecal | Urinary | |
| Material | (a) | (b) | (c) | (d) | (e) | (f) |
| Formyl | | | | | | |
| Lysine | — | (94) | — | — | — | — |
| Protein (lactalbumin) | 28 | — | 77 | — | — | — |
| Acetyl | | | | | | |
| Lysine | 0 | (100) | 50 | 18 | 18 | 86 |
| Protein (BPA) | 15 | — | 67 | — | — | — |
| Propionyl | | | | | | |
| Lysine | 0 | (0) | 0 | 19 | 41 | 60 |
| Protein (lactalbumin II) | 2 | — | 43 | (−16) | 41 | 84† |
| Control protein (BPA) | 91 | — | 85 | 14 | 1 | 100 |
| Heat-damaged protein (BPA) | 24 | — | 13 | 66 | 4 | 83 |

BPA, bovine plasma albumin.

* For the amino acids the zero values were based on the failure to find free lysine by thin-layer chromatography; for the proteins the direct FDNB-available lysine values were taken as the measure of unreacted lysine.

† This value is the sum of 'c' and 'e' alone, without the negative value for 'd' subtracted.

The first general conclusion from this study is that the formation of amide linkages between the ε-$NH_2$ groups of the lysine units and some other molecule containing carboxyl groups may well be a cause of nutritional damage in heated proteins, particularly since the biological activity of ε-*N*-acetyl-L-lysine, the only model material previously tested, is a special case in view of the presence of a specific kidney enzyme for which it is a substrate.

The second conclusion is that the metabolism of the lysine in propionyl lactalbumin is different from that in our example of a heat-processed protein. The essential changes that occurred in heating the pure protein (BPA) may still be condensations between carboxyl and ε-$NH_2$ lysine groups but, if so, the products are largely indigestible;

with the heat-processed protein there was no evidence of lysine-containing compounds being absorbed and re-excreted in the urine. This is not surprising if indigestible cross-linkages between peptide chains are involved.

We are grateful to Dr V. H. Booth, who carried out the determinations of total and residual lysine referred to in the paper and will be discussing these procedures in detail elsewhere. The elementary analyses were kindly carried out in the University Chemical Laboratory, and Dr R. C. Campbell supervised the statistical analyses. One of us (J. B.) was in receipt of a Broodbank Fellowship from the University of Cambridge.

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