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Some Aspects of the Destruction of Lysine under Conditions of Acid and Enzymic Hydrolysis of Protein Materials Containing Carbohydrates

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The injurious effects of dry heat on the nutritive value of many proteins has been repeatedly demonstrated (e.g. Morgan, 1931; Block, Jones & Gersdorff, 1934; Greaves, Morgan & Loveen, 1938). Greaves *et al.* (1938) reported that heating at 130° for 8 hr. produced only a slight decrease in the digestibility of dry casein but considerably reduced its nutritive value for rats. They also observed that growth of the rats could be restored by supplementing the heated casein with lysine. Block *et al.* (1934), using a chemical method for lysine assay, had previously observed that after acid hydrolysis as much lysine could be isolated from the heat-treated casein as from the untreated. They postulated that when a protein is heated in the dry state the free carboxyl groups of dicarboxylic amino acids might react with the ϵ -amino groups of lysine, forming a new peptide linkage which is resistant to enzymic digestion, but not to acid hydrolysis. Eldred & Rodney (1946), using the decarboxylase technique of Gale (1945) for the assay of lysine, confirmed the observations of Block *et al.* (1934). In contrast with protein-bound lysine, free lysine is remarkably stable to dry heat in the presence and absence of other free amino acids (Evans & Butts, 1948).

A second, different type of destruction of protein-bound lysine has been observed when proteins are heated with carbohydrates (Block *et al.* 1946; Clandinin, Cravens, Elvehjem & Halpin, 1947; Evans & Butts, 1948). Patton, Hill & Foreman (1948*a, b*), using a microbiological assay for the estimation of the amino acids, demonstrated that carbohydrates are concerned in the destruction of lysine. Patton & Hill (1948) postulated that the

destruction in this case was due to compound-formation between reducing sugars and those amino acid residues possessing free functional nitrogen groups, and that the chemical bond formed was not split by acid hydrolysis. On heating proteins with carbohydrate, or on heating carbohydrates alone for long periods, a brown colour appears and after long heat treatment a chocolate-brown precipitate (humin) forms. Mohammad, Contrat & Olcott (1949), studying the progressive disappearance of the free amino groups in bovine serum albumin, concluded that these groups were involved in the browning reaction. Many workers (e.g. Stevens & McGinnis, 1947; Patton & Hill, 1948) have demonstrated that lysine can also react with carbohydrates.

The studies mentioned above were all conducted in the absence of acid. The behaviour of free and bound lysine in acid solution is also of interest since amino acid analysis of proteins usually involves acid hydrolysis. Tristram (1939) studied the effect of refluxing amino acids with 8*N*-sulphuric acid in the presence and absence of carbohydrates, and concluded that arginine was significantly affected, whereas lysine was not. An interaction between amino acids and reducing sugars was claimed by Maillard (1912), and Roxas (1916) suggested that furan compounds were responsible for this Maillard reaction. Scallet & Gardener (1945) showed that aqueous solution of glucose became highly coloured when heated for several hours at 100° owing to the formation of 5-hydroxymethylfurfuraldehyde, followed by its polymerization to form humin substances. Wolfrom, Shuetz & Cavalieri (1948, 1949) and Wolfrom, Cavalieri & Cavalieri (1947) also suggested that 5-hydroxymethylfurfuraldehyde reacted with the free amino groups of amino acids. If pentoses were substituted for glucose, then the

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furfuraldehyde formed reacted with the free amino groups. Gottschalk & Partridge (1950) were the first to demonstrate qualitatively a reaction between an amino acid and 5-hydroxymethylfurfuraldehyde, but no quantitative data were presented. It has been shown that this Maillard reaction between amino acids and sugars causes the destruction of some free amino acids such as lysine, tryptophan, histidine (Patton *et al.* 1948*a*), methionine (Evans & Butts, 1949*a*), threonine and phenylalanine (Friedman & Kline, 1950); leucine, valine and isoleucine are not appreciably affected (Patton *et al.* 1948*b*; Evans & Butts, 1949*a, b*; Friedman & Kline, 1950).

This destruction of essential amino acids on heating in the presence of carbohydrates is of importance not only in food processing but also in the analysis of foodstuffs, which are usually hydrolysed with 6*N*-hydrochloric acid without prior removal of carbohydrates. These considerations led us to investigate further the destruction of lysine in the presence of carbohydrates and 5-hydroxymethylfurfuraldehyde under acid conditions.

MATERIALS AND METHODS

L-Lysine. L-Lysine was used throughout and was obtained as the monohydrochloride from Nutritional Biochemicals Corporation, New York.

5-Hydroxymethylfurfuraldehyde (HMF). This was prepared as described by Haworth & Jones (1944). The crude product was purified by vacuum distillation and collection of the fraction that distilled at 112° and at a pressure of 0.02 mm. Hg.

Humin from starch. Rice starch (150 g.) was made into a thin paste with water and mixed with 6*N*-HCl (400 ml.). The suspension was warmed on a boiling-water bath until the starch dissolved completely. After refluxing for 24 hr. the dark humin was filtered off under mild suction and washed repeatedly until the filtrate was free from HCl. The residue was dried at 105° to constant weight, giving 16.7 g. of a chocolate-brown powder.

Determination of lysine

Hydrolysis and preparation of sample for lysine assay. The hydrolysis was carried out by refluxing with 6*N*-HCl for 24 hr. at 105–110°. The hydrolysates were concentrated under vacuum in a desiccator over solid NaOH and CaCl₂. The dry residues were dissolved in water, the pH adjusted to 1.0 with HCl and the resulting solutions filtered if necessary and diluted as required, after adjustment of the pH to 5.0 with NaOH.

Estimation. Lysine was estimated by the decarboxylase method of Gale (1945), with acetone-dried powders of *Bacterium cadaveris* (N.C.T.C. 6578) as a source of lysine decarboxylase.

Effect of concentration of sodium chloride on the activity of lysine decarboxylase. There are no available data on the effect of NaCl on lysine decarboxylase, although Gale & Epps (1944) showed that (NH₄)₂SO₄ inhibited the decarboxylation and mentioned that NaCl gave a similar effect.

Table 1. *Effect of concentration of sodium chloride on the activity of lysine decarboxylase*

The experimental system contained 1.5 ml. of L-lysine solution (10 μmoles/ml.), 1.0 ml. of 0.1*M*-acetate buffer (pH 5.0), 0.5 ml. of lysine decarboxylase preparation (50 mg./ml.), 0.5 ml. of NaCl solution to give the indicated concentration and water to make the total vol. 3.5 ml. Gas phase air, temp. 37°.

Concn. of NaCl (M)	Rate of liberation of CO ₂ (μl./5 min.)	Inhibition by NaCl (%)
0	320	—
0.05	302	6
0.10	278	13
0.20	241	25
0.30	210	34
0.40	181	44
0.50	150	53

The neutralized hydrolysates usually contain relatively large amounts of NaCl. The effect of this compound on the activity of the lysine decarboxylase was examined. Our results (Table 1) indicate that concentrations of 0.05*M* and above significantly inhibit decarboxylation, though even at 0.5*M*-NaCl the inhibition was only 53%. In all determinations the final concentration of NaCl in the assay system was less than 0.05*M*.

Other analytical methods

Determination of 5-hydroxymethylfurfuraldehyde. HMF in buffer solutions was determined spectrophotometrically by measuring the absorption at 285 mμ with the Beckman model DU spectrophotometer. Measurements were made relative to a blank cell containing only the corresponding buffer and the concentration of HMF was estimated by comparison with the absorption of a standard solution.

Determination of nitrogen. The nitrogen content of the humin was estimated by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943); the NH₃ was distilled in the apparatus of Markham (1942) and trapped in the boric acid reagent of Conway & O'Malley (1942).

Determination of pH. All measurements were made on a Beckman pH meter.

RESULTS

Preliminary experiments showed that on heating lysine with a variety of hexoses and pentoses the amount of lysine destroyed was related to the amount of humin formed. It seemed possible that humin was responsible for the destruction of lysine, and in order to attempt to correlate results with those obtained in the preliminary experiments tests were carried out at various pH values. When lysine was heated at 100° with humin for 2 hr. at pH 6.8 approximately 50% of the lysine was lost (Table 2). The loss of lysine decreased with the pH and became almost negligible at pH 1.0. Lysine losses in the presence of carbohydrates are therefore associated with formation of humin. The lysine which was lost on heating with humin at pH 6.8 could not be recovered by subsequent extraction of

the precipitate with 0.1N-HCl at room temperature; this suggested that the loss was due to chemical combination of lysine with the humin rather than to physical adsorption.

Table 2. *Effect of heating rice-starch humin with lysine at various pH values*

Lysine monohydrochloride (178 μ moles) was mixed with 1.6 g. of rice-starch humin and suspended in 25 ml. of water; the pH was adjusted with HCl as required. The suspension was heated at 100° for 2 hr. and filtered after cooling to room temp. The residues were washed three times with approximately 5 ml. of water adjusted to the appropriate pH with HCl; the washings were pooled with the original filtrate and the pH was measured. No pH change greater than 0.05 was observed in any of the experiments. The solutions were concentrated under vacuum and lysine was determined. Lysine determinations were carried out on the untreated solutions and in all cases gave values of 178 μ moles.

pH	Lysine recovered (μ moles)	Loss of lysine (%)
1.0	175	3
2.0	170	6
3.3	158	13
4.8	133	26
5.1	131	27
6.8	88	51

It thus seemed probable that the losses of lysine might be minimized by filtering off the humin at acid pH before neutralizing. To test this, mixtures of lysine and starch and of edestin and starch were refluxed with 6N-HCl.

The losses after filtering at pH 5.0 were 80 % or more (Table 3) both with free and protein-bound lysine. In both cases these losses could be considerably reduced by carrying out the filtration at pH 1.0, though the losses at this pH were still over 20 %. In all experiments the losses observed when lysine was heated with starch were much higher than those observed with hexoses or pentoses.

Interaction of glucose and 5-hydroxymethylfurfuraldehyde with free and protein-bound lysine under various conditions. The two preceding experiments were concerned mainly with the interaction of lysine with simple or complex carbohydrates. The interaction between protein-bound lysine and a simple carbohydrate was next studied. Four different types of experiments were conducted with fibrin as a source of protein-bound lysine and glucose as the carbohydrate (Table 4). The recovery of lysine after heating fibrin with 6N-HCl for 48 hr. was determined (Expt. 1) and taken as a measure of its lysine content. Addition of glucose during the last 24 hr. of the treatment (under

Table 3. *Effect of removing humin at pH 1.0 and 5.0 and lysine recovery*

Lysine (178 μ moles) or edestin (1 g.) was mixed with 10 g. of starch and refluxed for 24 hr. with 40 ml. of 6N-HCl. The hydrolysates were dried and the residues dissolved in 50 ml. of water; the humins were filtered off at pH 1.0 or pH 5.0. The residues were washed three times with 5.0 ml. of water adjusted to the appropriate pH; the washings were pooled with the original filtrate and concentrated again. The dry residue was dissolved in 25 ml. of water and lysine estimated. The value with edestin + HCl was taken as a true measure of lysine content of the edestin in calculating the loss.

System	Humin filtered off at pH 5.0		Humin filtered off at pH 1.0	
	Lysine recovered (μ moles)	Lysine loss (%)	Lysine recovered (μ moles)	Lysine loss (%)
Lysine + HCl	176	—	176	—
Lysine + starch + HCl	35	80	132	25
Edestin + HCl	151	—	151	—
Edestin + starch + HCl	12	92	120	21

Table 4. *Interaction of glucose with protein-bound lysine under various conditions*

The experimental systems initially contained the components given in column (1); after 24 hr. hydrolysis treatment, the components in column (2) were added and the hydrolysis treatment continued for a further 24 hr. The final vol. and concn. of acid were the same at the end of each experiment. In calculating the loss of lysine, the value for lysine obtained in Expt. 1 was taken as a true measure of lysine content.

Expt. no.	Components present in 1st stage (0-24 hr.)	Components added for the second stage (24-48 hr.)	Lysine recovered (μ moles)	Lysine loss (%)	Total humin produced (mg.)	Total N of humin (mg.)
1	Fibrin (300 mg.) + 6N-HCl (15 ml.)	—	180	—	5	0.33
2	Fibrin (300 mg.) + 6N-HCl (15 ml.)	Glucose (1.5 g.)	177	2	226	1.98
3	Fibrin (300 mg.) + glucose (1.5 g.) + 6N-HCl (15 ml.)	—	152	15	347	3.16
4	Fibrin (300 mg.) + glucose (1.5 g.) + H ₂ O (6 ml.)	10N-HCl (9 ml.)	49	73	121	5.74

which conditions complete hydrolysis of the fibrin would have occurred, thus giving rise to free lysine) (Expt. 2) caused only a slight loss of lysine; a somewhat higher loss (15%) was observed when glucose was present from the beginning (Expt. 3). The results therefore indicate that glucose does not react to any great extent with free lysine but does react with protein-bound lysine. When an aqueous solution containing glucose and fibrin was refluxed at neutral pH, under which conditions little, if any, hydrolysis of the protein occurred and then subsequently hydrolysed with HCl in the usual way, the loss of lysine was 75% (Expt. 4, Table 4). These results would therefore indicate that protein-bound lysine reacts very readily with glucose at least at a neutral pH. The amount of humin formed and also its nitrogen content were measured in each case. The amounts of humin formed bore no obvious relationship to the loss of lysine, but the amount of nitrogen appearing in the humin increased with the loss of lysine. This would suggest that the loss of lysine is associated with the incorporation of lysine nitrogen into the humin.

Scallet & Gardener (1945) suggested that when glucose is heated for a long time at 100° it is converted into 5-hydroxymethylfurfuraldehyde which in turn polymerizes to form humin-like substances. The reaction of free and protein-bound lysine with the aldehyde was therefore investigated.

When the interaction with protein-bound lysine was examined, the results (Table 5) were similar to those obtained with fibrin and glucose, i.e. the loss of lysine was much greater at a neutral pH than in acid (cf. Expts. 2 and 3), Table 5. No losses of

lysine were observed at an acid pH when free lysine and 5-hydroxymethylfurfuraldehyde were subjected to the same treatment (Expt. 1).

Effect of incubating 5-hydroxymethylfurfuraldehyde with free lysine at 37° at various pH values. Gottschalk & Partridge (1950) found that 5-hydroxymethylfurfuraldehyde reacts with lysine at pH 8.0 and at 37°. This led us to investigate the effect of incubating free and bound lysine with the aldehyde at this lower temperature. When fibrin and 5-hydroxymethylfurfuraldehyde were incubated at 37° for 132 hr. and subsequently hydrolysed, 76% of the lysine was lost (Expt. 4, Table 5). When excess of the aldehyde was removed by centrifuging and repeated washing before hydrolysis treatment, the loss was much smaller (33%), though still substantial (Expt. 5).

At this lower temperature and at a neutral pH some interaction between free lysine and 5-hydroxymethylfurfuraldehyde was observed (Expt. 6) and when solid lysine hydrochloride was allowed to react with the aldehyde the losses were larger, amounting to 34%. The high loss with lysine hydrochloride might have been due to the more acid pH and the effect of pH upon the interaction of the aldehyde with lysine was therefore examined.

The results are given in Table 6; they indicate that, by starting from an acid pH, the destruction of lysine by 5-hydroxymethylfurfuraldehyde decreases with increasing pH, reaches a minimum between pH 4.0 and 5.0 and then increases with increasing pH. It was suspected that the lower loss of lysine over this middle range might be due to the destruction of the aldehyde. The behaviour

Table 5. *Interaction of 5-hydroxymethylfurfuraldehyde with free and protein-bound lysine under various conditions*

The experimental system contained 300 mg. of HMF and 300 mg. of fibrin or 46 μ moles of lysine. In Expt. 5 after pretreatment the excess of HMF was removed by centrifuging and by repeated washing (three times with approximately 5 ml. of water). In Expt. 7 solid lysine hydrochloride was mixed with HMF (liquid) and incubated. The components given in column (1) were mixed and pretreated before hydrolysis as given in column (2). After pretreatment the products of Expts. 1 and 3 were hydrolysed for a further period of 24 hr.; to each of the products of Expts. 2 and 4 15 ml. of 10N-HCl was added, and to the products of Expt. 5 24 ml. of 10N-HCl was added and hydrolysis treatment was carried out for 24 hr. In calculating the losses of lysine, the value for lysine content of fibrin was taken from Expt. 1, Table 4.

Expt. no.	System	Pretreatment before acid hydrolysis	Hydrolysis treatment for 24 hr.	Lysine recovered (μ moles)	Loss of lysine (%)
1	Lysine + HMF + 6N-HCl (25 ml.)	Refluxed for 24 hr.	Hydrolysis continued	46	0
2	Fibrin + HMF + H ₂ O (10 ml.)	Refluxed for 24 hr.	With 10N-HCl (15 ml.)	84	52
3	Fibrin + HMF + 6N-HCl (25 ml.)	Refluxed for 24 hr.	Hydrolysis continued	130	26
4	Fibrin + HMF + H ₂ O (10 ml.)	At 37° for 132 hr.	With 10N-HCl (15 ml.)	42	76
5	Fibrin + HMF + H ₂ O (10 ml.)	At 37° but excess of HMF removed	With 10N-HCl (25 ml.)	118	33
6	Lysine + HMF + H ₂ O (25 ml.)	At 37° for 48 hr.	No hydrolysis	39	13
7	Solid lysine-HCl + HMF	At 37° for 48 hr.	No hydrolysis	30	34

of the aldehyde when incubated for a considerable time over the pH range 1.0–8.0 was therefore studied.

Effect of incubating 5-hydroxymethylfurfuraldehyde alone at 37° at various pH values. The results (Table 7) indicate that 5-hydroxymethylfurfuraldehyde is stable over the pH ranges 1.0–4.0 and 7.0–8.0, but very unstable over the range 4.6–6.6, the maximum instability being observed at pH 5.6. It was found that the disappearance of the aldehyde was associated with the appearance of material absorbing light at 252 m μ . The formation of this material reached a maximum at the pH that was optimum for destruction of 5-hydroxymethylfurfuraldehyde. When this material absorbing light at 252 m μ was formed by the incubation of 5-hydroxymethylfurfuraldehyde at pH 5.6, the alde-

Table 7. *Effect of incubating 5-hydroxymethylfurfuraldehyde for different times at various pH values*

HMF (0.5 ml.) was dissolved in water (1 l.). 1.0 ml. of this solution was mixed with 99 ml. of the appropriate buffer solution and incubated at 37°. Samples were withdrawn after various periods and the concentration of HMF was measured spectrophotometrically. In calculating the disappearance of HMF, the values at zero time in various buffers were determined (column 2). For buffer solution (pH 1.0). 0.1 N-HCl was used, and for the other range of buffers (pH 2.2–8.0) mixtures of 0.1 M-citric acid and 0.2 M-Na₂HPO₄ in different proportions were used. Phthalate buffer solutions could not be used here as they absorb light at 285 m μ .

Table 6. *Effect of incubating 5-hydroxymethylfurfuraldehyde with free lysine at various pH values*

The various buffer solutions were made as follows: for pH 1.0, 0.1 N-HCl; pH 1.4–2.2, 0.2 N-HCl + 0.2 M-KCl mixture in different proportions; pH 3.4, 0.2 M-KCl + 0.067 M-potassium hydrogen phthalate; pH 4.4–5.6, 0.1 N-acetic acid + 0.1 M-sodium acetate; pH 6.0–8.0, 0.067 M-Na₂HPO₄ + 0.067 M-KH₂PO₄. The experimental system contained 1.0 ml. of lysine (1 m-mole/ml.), 1.0 ml. of HMF (2.5 m-mole/ml.), 40 mg. of NaF and 8.0 ml. of the appropriate buffer solution. The mixtures were incubated at 37° for 132 hr., after which time the pH values were checked and lysine was estimated. The losses were calculated from the values obtained by analysis of the mixture at zero time (column 2).

pH	Lysine recovered (m-moles)		Lysine loss (%)
	Before incubation	After incubation	
1.0	1.00	0.64	36
1.4	0.99	0.79	20
1.8	0.99	0.82	17
2.2	0.98	0.86	12
3.4	0.99	0.94	5
4.4	1.00	0.97	3
4.8	0.99	0.97	2
5.2	0.99	0.95	4
5.6	0.99	0.94	5
6.0	1.00	0.92	8
7.0	1.00	0.88	12
8.0	0.99	0.84	15

hyde was not reformed on subsequent incubation at pH 1.0, indicating that the transformation was not reversible.

DISCUSSION

Roxas (1916) suggested that the interaction of carbohydrates with proteins involved a preliminary formation of 5-hydroxymethylfurfuraldehyde from the carbohydrate and its subsequent reaction with free amino groups (reactions 1 and 2).

Scallet & Gardener (1945) demonstrated that reaction (1) occurs under both acid and neutral conditions at temperatures of 100° and above. As far as reaction (2) is concerned, Gottschalk & Partridge (1950) have shown that at a pH of 8 and at 37° 5-hydroxymethylfurfuraldehyde reacts with free amino acids, but this was the only information

available. In the present studies the destruction of lysine by 5-hydroxymethylfurfuraldehyde on incubating at 37° has been examined over a pH range 1.0-8.0 and it has been observed that the destruction of lysine is at a minimum over the pH range 4.0-6.0. This is precisely the range over which 5-hydroxymethylfurfuraldehyde is least stable, suggesting that the destruction of free lysine requires its participation.

It is known that under acid conditions 5-hydroxymethylfurfuraldehyde is rapidly converted into laevulinic and formic acids (Karrer, 1938). No loss of lysine occurred when it was refluxed with 6N-HCl containing 5-hydroxymethylfurfuraldehyde. This indicates that an interaction between the aldehyde and free lysine is unlikely to occur during the acid hydrolysis of foodstuffs. Although no loss of free lysine occurred when it was refluxed under these conditions, a considerable loss of lysine was observed when proteins were hydrolysed under the same conditions. These results are compatible with the idea that reaction (2) can occur with protein-bound lysine and that reactions (1) and (2) can account for the losses of lysine observed. It is apparent that, even after acid hydrolysis, the loss of lysine is quite appreciable (15%) when carbohydrate is present; consequently results obtained after acid hydrolysis under these conditions must be interpreted with caution. The results with fibrin and 5-hydroxymethylfurfuraldehyde alone at 37° indicate that even under conditions such as might be used for enzymic hydrolysis the losses are still appreciable.

The loss of lysine seems to be associated with the humin formed during this treatment, as it was observed that the loss of lysine increased with the amount of nitrogen appearing in the humin. The destruction of lysine can also occur by interaction of the amino acid with the humin; for example, when free lysine was mixed with humin and boiled at 100° a considerable loss was observed. This can be minimized considerably if the humin is filtered off at pH 1.0 rather than at pH 5.0.

SUMMARY

1. Lysine was destroyed when heated with starch, the loss being related to the amount of humin produced.

2. The loss of lysine on heating with humin increased with increasing pH. Maximum loss occurred at pH 6.8 and negligible loss at pH 1.0.

3. After acid hydrolysis removal of humin at pH 1.0 instead of pH 5.0 reduced the loss of lysine considerably.

4. Free and protein-bound lysine were destroyed on heating with glucose or 5-hydroxymethylfurfuraldehyde. Protein-bound lysine reacted more

readily with glucose and 5-hydroxymethylfurfuraldehyde at a neutral than at an acid pH.

5. Free and protein-bound lysine reacted with 5-hydroxymethylfurfuraldehyde at 37° and more readily at a neutral than at an acid pH. 5-Hydroxymethylfurfuraldehyde was found to be unstable at 37° over the pH range 4.6-6.6.

6. The possible pathway by which lysine reacts with glucose is discussed.

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