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The Irreversible Combination of Formaldehyde with Proteins

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The reaction of formaldehyde with proteins has been of interest for many years, owing to its industrial applications including tanning and plastics manufacture. Until recently the type of linkage formed was largely a subject for speculation rather than experiment. However, recent work has shed light on the details of the reaction. The literature has been adequately reviewed by French & Edsall (1945). Middlebrook & Phillips (1942, 1947) showed that at elevated temperatures the disulphide S of wool combines with formaldehyde, and that the reaction can be completely reversed by distillation with dilute phosphoric acid; it became apparent, however, that formaldehyde which combines with other groups cannot be completely removed in this way. Nitschmann, Hadorn & Lauener (1943) showed for casein that this method gave low results unless water was added, and a second distillation carried out; even so, the recovery of formaldehyde was not quantitative, and similar results were likewise obtained when 2N-sulphuric acid was used instead of dilute phosphoric acid. When the distillation was continued to dryness, the results were unreliable, owing to the destruction of the protein, and the production of bisulphite-binding substances.

Wormell & Kaye (1945) and Fraenkel-Conrat, Cooper & Olcott (1945b) showed that the amide groups of proteins would combine with formaldehyde under acid conditions, and the latter authors found that the reaction was slow and greatly dependent upon formaldehyde concentration and reaction temperature. The present work was carried out to determine the effects of concentration of formaldehyde and pH on the amount of combined formaldehyde, which was stable to boiling dilute phosphoric acid and known as irreversibly combined formaldehyde.

METHODS

Materials

The wool used was a virgin Cape wool of about 64's quality, from which the tip ends had been removed, cleaned by successive extraction with ethanol, diethyl ether, and finally distilled water. Dark human hair was used for the supercontraction experiment. It was extracted by diethyl ether, any temporary set removed by allowing it to float freely in distilled water, and then carefully dried. Details of other protein preparations are as follows: casein, prepared from separated milk by precipitation with lactic acid (Van Slyke & Baker, 1918), N=15.7%, amide N=1.58%; collagen, sample from Dr D. Jordan Lloyd; edestin, sample from Roche Products, Ltd.; gelatin, Coignet 'Extra', amide N=0.45%; gliadin, sample from Prof. A. C. Chibnall; glycinin, prepared from fat-free soya meal by the method of

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Jones & Moeller (1928), $N=17.47\%$; fully methylated wool, prepared by Dr H. Lindley (see Blackburn, Carter & Phillips, 1941); haemoglobin (horse CO-haemoglobin), prepared and heat-coagulated according to the method of Adair & Adair (1934), $N=17.2\%$, $Fe=0.33\%$, amide $N=1.08\%$; ovalbumin, sample of unknown origin, coagulated, dried and powdered. The proteins were extracted by diethyl ether, with the exception of the edestin and gelatin. The collagen was acetone-extracted. They were all air-dried, the moisture contents measured, and all determinations calculated on dry weight of protein.

Treatment and analytical methods

Formaldehyde treatment of hair. Bundles of hair (1 g.), tied by cotton thread, were boiled under reflux for 1 hr. in 50 ml. of 1% (w/v) formaldehyde solution, buffered in (i) borax adjusted with NaOH to pH 10.1 (final concentration of borax, 0.05M); (ii) NaH_2PO_4 adjusted with NaOH to pH 6.65 (final concentration of NaH_2PO_4 , 0.1M); (iii) sodium acetate adjusted with HCl to pH 1.00 (final concentration of sodium acetate 0.2M). The formalized hair was washed overnight in distilled water. Some of the hairs were boiled in 250 ml. 0.1M- H_3PO_4 until the volume of the latter was c. 10 ml., then washed and dried. No change of length of the hairs took place during any of these treatments.

Deamination. Hair and other proteins were deaminated in a mixture of $NaNO_3$ and dilute acetic acid (Van Slyke's reagent), according to the technique of Speakman (1934).

Deamidation. Proteins were partly deamidated by the following techniques: (i) in a solution of cetylsulphuric acid and HCl (Steinhardt & Fugitt, 1942); (ii) in a solution of 1% NaOH for 40 hr. at 45° (Wormell & Kaye, 1945); (iii) in a solution of $NaNO_3$ and acetic acid, containing 2N-HCl for 24 hr. at room temp. (Plimmer, 1925; Fraenkel-Conrat, Cooper & Olcott, 1945a). This method would also deaminate the proteins, and remove the guanidino groups.

Estimation of tyrosine. The intensity of the colour given by the action of α -nitroso- β -naphthol on tyrosine was measured in the Spekker absorptiometer using green filters (Thomas, 1944). It was observed that cystine suppressed the colour. When the ratio of cystine to tyrosine was the same as that in wool, the value was lowered by 2.5%. In the case of wool, therefore, a correction factor was applied.

Bisulphite-stable fraction of the combined formaldehyde of formalized casein. Casein (1.5 g.) was boiled under reflux for 1 hr. in 5 ml. of pH 1 buffer and 5 ml. of 8% (w/v) formaldehyde; 75 ml. of 2.4% (w/v) $NaHSO_3$ solution were added, the mixture boiled under reflux for 20 min., left overnight, filtered, the precipitate washed, and the formaldehyde combined with the $NaHSO_3$ determined. The difference between the amount of formaldehyde taken and the amount in combination with the $NaHSO_3$ is, therefore, equal to the amount in combination with the casein and stable to boiling $NaHSO_3$.

Estimation of irreversibly combined formaldehyde

Macro method. Approx. 1 g. of protein was weighed into a small round-bottomed flask fitted with an air condenser having a ground-glass joint; 5 ml. of buffer (Na acetate + HCl, pH 0.46, 1.0, 2.0, 3.9; final concentration of acetate 0.4N) together with 5.0 ml. of formaldehyde solution were added, the joint being lightly greased with petroleum jelly. The mixture was boiled under reflux for 1 hr., cooled, then quickly transferred, together with any washings, to a 250 ml.

flask containing 100 ml. of 0.3M- H_3PO_4 . The liquid was then distilled, down to a vol. of c. 5 ml., into a solution of $NaHSO_3$ (Nitschmann & Hadorn, 1941). Water was added to the flask and the distillation repeated, making a total of 4 distillations. Blanks containing known amounts of formaldehyde, without the addition of protein, were also distilled, and the amounts of formaldehyde distilled over were compared with direct estimations obtained by adding 5.0 ml. of formaldehyde solution directly to the $NaHSO_3$ solution. A complete recovery of the formaldehyde was obtained. If the protein frothed unduly, anhydrous Na_2SO_4 (2 g.) was added to the distillation flask. All the ground-glass joints of the distillation apparatus were lightly greased. The solutions were left overnight, and the excess $NaHSO_3$ titrated with 0.1N- I_2 using starch as indicator. Ethanol was then added to prevent oxidation, the $NaHSO_3$ -formaldehyde compound decomposed by the addition of 1.0N- Na_2CO_3 , and the liberated $NaHSO_3$ titrated with 0.1N- I_2 (1 ml. 0.1N- $I_2 \equiv 0.0015$ g. formaldehyde; Highberger & Retzsch, 1938).

Semimicro method. When only small amounts of protein were available, the method was modified. The protein (c. 40 mg.) was weighed in a glass tube sealed at one end, about 12 cm. in length and 7 mm. in internal diameter; 0.18 ml. buffer and 0.18 ml. formaldehyde solution were introduced by means of 2 Conway pipettes, which had been converted into burettes having capillary extensions with right-angle bends attached to their tips, the flow of liquid being controlled by surface tension. The tube was immediately sealed in a blowpipe flame, then placed in an oven at 100° for 1 hr., and occasionally shaken. After this, it was broken in half and quickly dropped into 100 ml. of 0.3M- H_3PO_4 . The distillation was repeated as before. Blank distillation of known amounts of formaldehyde gave complete recovery.

Free amino nitrogen. This was determined manometrically according to the technique of Peters & Van Slyke (1932), but in a blacked-out chamber (Fraenkel-Conrat, 1943).

Free amino, guanidino, and part of the amide nitrogen. The determinations were made in the Van Slyke microvolumetric apparatus (Plimmer, 1917), in the presence of 2N-HCl for 22 hr. in a blacked-out chamber (Plimmer, 1925; Fraenkel-Conrat *et al.* 1945a).

Amide nitrogen. Protein (0.25 g.) was refluxed with 5N-HCl (5 ml.) for 4 hr., filtered, then diluted to 25 ml.; 5 ml. samples were made just alkaline to bromocresol green in the semimicro-Kjeldahl apparatus. 5 ml. of 2% borax solution were added to bring the pH between 8 and 9 (Lindley, 1946). NH_3 was distilled into a saturated solution of boric acid, and titrated with N/70-HCl, using a mixed indicator of bromocresol green and methyl red (Ma & Zuazaga, 1942).

Other methods. Total nitrogen was determined by the micro-Kjeldahl method (Chibnall, Rees, & Williams, 1943a) and cystine by the method of Shinohara (1935). Two-dimensional paper chromatograms were made according to the technique of Consden, Gordon & Martin (1944), using water-saturated collidine as one solvent, and water-saturated phenol over 0.3% NH_3 solution in an atmosphere of coal gas as the other.

RESULTS

The effect of formaldehyde on the supercontraction of hair (Speakman, 1936) after boiling for 30 min. in 5% (w/v) sodium bisulphite solution is shown in Table 1 (cf. Middlebrook & Phillips, 1942; Stoves, 1943).

Table 1. *Supercontraction of formaldehyde-treated hair, after boiling in 5% NaHSO₃ for 30 min.*

pH of formaldehyde	Supercontraction		
	NaHSO ₃ only (a) (%)	Distilled in 0.1 M-H ₃ PO ₄ before NaHSO ₃ (b) (%)	(b-a) (%)
10.1-9.4	3.3	14.0	10.7
6.7	19.8	32.3	12.5
1.0	5.0	28.6	23.6
Untreated	28.0	27.5	—
Deaminated			
Untreated	28.5	—	—
1.0	7.5	—	—
Deaminated (Steinhardt & Fugitt, 1942)			
Untreated	Dissolved immediately	—	—
1.0	48.2	43.7*	—

* All the results are expressed as the mean of four determinations, except *, which is one determination only.

In view of the instability to bisulphite of cross linkages between amino groups (Fraenkel-Conrat *et al.* 1945*b*), it is unlikely that these contribute to the diminished supercontraction shown by formaldehyde-treated hair in boiling sodium bisulphite solution. This is confirmed by the fact that deaminated, as well as untreated fibres, which have been boiled in formaldehyde at pH 1, fail to supercontract on boiling in bisulphite solution (Stoves, 1944). On deamidation by the Steinhardt & Fugitt (1942) method (residual amide N, 0.4%), it is noticed that formaldehyde treatment confers no stability to supercontraction, although it does prevent the hair from being immediately dissolved in the boiling bisulphite solution. From these results, it is suggested that the values obtained in column 4 are due to cross linkages formed between the amide groups of glutamine and the guanidino groups of arginine (Wormell & Kaye, 1945; Fraenkel-Conrat *et al.* 1945*b*, 1946). From evidence to follow it is suggested that asparagine combines with formaldehyde, but it is unlikely to contribute to cross-linkage formation. The amount of formaldehyde in combination with casein which is stable to boiling bisulphite solution is equal to 3.67%, equivalent to 1.71% amide N, if one amide group combines with 1 mol. of formaldehyde (actual amide N, 1.58%). When wool treated with formaldehyde at pH 1 was hydrolyzed and run on a two-dimensional chromatogram, no tyrosine spot was found (Middlebrook & Phillips, 1947). In order to ascertain if formaldehyde under these conditions does react with combined tyrosine, a sample of wool was treated with formaldehyde at pH 1, distilled in 0.3M-phosphoric acid, hydrolyzed for 4 hr. in 5N-hydrochloric acid, and tyrosine determined by the method of Thomas (1944). The tyrosine content of untreated wool was 5.43%, and of treated 5.39%. It is unlikely, therefore, that formaldehyde has combined with tyrosine

under these conditions, but when liberated on hydrolysis it may be expected to combine with free tyrosine, histidine, etc. (Neuberger, 1944).

In order to determine irreversibly combined formaldehyde, distillations were carried out in 2N-sulphuric acid until fumes appeared in the distillation flask. Confirming the observations of Nitschmann *et al.* (1943), more efficient removal of formaldehyde was obtained by repeated distillation with dilute phosphoric acid. Undue hydrolysis of protein was avoided under these conditions.

The effect of formaldehyde concentration on the amount irreversibly combined at pH 1 is illustrated in Fig. 1. At concentrations above 4%, most of the proteins examined combine irreversibly with a small additional amount of formaldehyde. The effect of pH on the amount of irreversibly combined formaldehyde from a 4% (w/v) formaldehyde solution is illustrated in Fig. 2. The values plotted in the two diagrams are the means of at least two determinations. The values of the minima of the curves in Fig. 2, most of which occur between pH 1 and 2, are compared with the aspartic acid contents of the proteins, assuming that 1 mol. of irreversibly combined formaldehyde is equivalent to 1 mol. of aspartic acid. These values are summarized in Table 2, and compared with recent values for the aspartic acid content of the corresponding proteins. The increase in the amount of irreversibly combined formaldehyde at pH 3.9 is probably due to combination with histidine, as these values are roughly proportional to the histidine content, if 1 mol. of formaldehyde combines with one histidine residue (cf. Neuberger, 1944). On increasing the concentration of formaldehyde above 4% (Fig. 1), a considerable amount of histidine may react with formaldehyde at pH 1 (Neuberger, 1944), explaining the values obtained for carboxyhaemoglobin and casein, both of which have a high histidine content. When samples of these

latter proteins were hydrolyzed, after first being distilled in 0.3M-phosphoric acid to remove the reversibly combined formaldehyde, they were found to give no spot for histidine on a two-dimensional chromatogram.

Fig. 1. Effect of formaldehyde concentration on amount of irreversibly combined formaldehyde produced at pH 1 and 100° for 1 hr. Protein concentration 10 g./100 ml. Final concentration of sodium acetate in buffered solution 0.2N.

The increases at pH 0.46 are presumably due to partial hydrolysis of the proteins, followed by irreversible combination of formaldehyde with the products of hydrolysis.

The role of amide nitrogen in the production of irreversibly combined formaldehyde

If the formaldehyde combined irreversibly between pH 1 and 2 is combined with asparagine amide groups, deamidation should prevent this combination. Proteins were ac-

cordingly deamidated by the Steinhardt & Fugitt (1942) technique. As these authors found it impossible to remove all the amide groups, those most easily removed were believed to arise from glutamine residues. It was also found that sufficient peptide hydrolysis had taken place to cause

Fig. 2. Effect of pH on amount of irreversibly combined formaldehyde produced in 4% (w/v) formaldehyde solution at 100° for 1 hr. Protein concentration 10 g./100 ml. Final concentration of sodium acetate in buffered solution, 0.2N.

an increase, rather than a decrease, in the amount of combined formaldehyde. Also, an increase in the time of treatment with the deamidating agent increased the amount of irreversibly combined formaldehyde without decreasing the amide N. Deamidation of wool in 1% NaOH solution

Table 2. Relation of irreversibly combined formaldehyde and the aspartic acid content of proteins

Protein	Irreversible formaldehyde* (%)	Aspartic acid calculated from treatment in		Aspartic acid from literature (%)
		4% formaldehyde (%)	8% formaldehyde (%)	
Carboxyhaemoglobin	2.63	11.6	16.5	10.3 (4) 9.3 (1) 10.8 (6)
Edestin	1.84	8.2	12.0	10.2 (7) 12.0 (2)
Casein	1.76	7.8	10.9	6.7 (1) 7.2 (6)
Glycinin	1.60	7.1	10.1	9.4 (7)
Ovalbumin	1.38	6.1	6.8	9.3 (6) 8.1 (2) 6.2 (7)
Gliadin	1.35	5.9	—	0.8 (7) 1.4 (1) 3.3 (6)
Wool	1.17	5.2	5.2	5.7 (8) 7.3 (8)
Gelatin	0.84	3.7	—	3.4 (3) 6.8 (2)
Silk	0.44	2.0	2.9	2.8 (6)

* Treatment in 4% formaldehyde.

- (1) Bailey, Chibnall, Rees & Williams (1943).

(2) Chibnall, Rees & Williams (1943b).

(3) Dakin (1920).

(4) Foster (1945).
- (5) Gordon, Martin & Synge (1941).

(6) Hao & Snell (1945).

(7) Jones & Moeller (1928).

(8) Speakman & Townsend (1937).

(Wormell & Kaye, 1945) was unsatisfactory, as only a small amount of amide N was removed: untreated wool, 1.15%; treated wool, 0.84%. Peptide hydrolysis took place, causing a higher value for irreversibly combined formaldehyde. In the case of wool, deamidation by the Plimmer (1925) technique was only slightly more successful: untreated wool, 1.15%; treated wool, 0.76%; untreated-wool cystine S, 3.25%; treated-wool cystine S, 0.53%. This treatment would also remove all the primary amino as well as the guanidino N.

In the case of carboxyhaemoglobin, no amide N was removed by the Plimmer technique: untreated protein, 1.08%; treated protein, 1.10%. The amount of irreversibly combined formaldehyde was approximately unchanged: untreated protein, 2.62%; treated protein, 2.74%.

The amide N is somewhat higher than the value obtained by Rees (1946) after hydrolysis in conc. HCl at 37°. From the information he gives, it is unlikely that decomposition of serine and threonine can account for more than 0.03% N, as the hydrolysis is only carried out for 4 hr.

As shown by Sanger (1945), 2:4-dinitrophenyl derivatives of amino-acids are remarkably stable to acid hydrolysis. A specimen of α -N-2:4-dinitrophenylasparagine was, therefore, prepared (Abderhalden & Blumberg, 1910), recrystallized and dried (m.p. 185°). A sample was treated with formaldehyde in aqueous acetone, containing a drop of conc. HCl, evaporated to dryness *in vacuo* several times with water, to remove excess formaldehyde, the residue distilled in 100 ml. 0.3M-H₃PO₄ and the formaldehyde collected in NaHSO₃ solution. If 1 mol. of formaldehyde is equivalent to 1 mol. of 2:4-dinitrophenylasparagine, then the amount of formaldehyde found was equivalent to 118% of the 2:4-dinitrophenylasparagine taken.

Asparagine combines with formaldehyde to form 6-hydroxytetrahydropyrimidine-4-carboxylic acid (Schiff, 1900; Cherbuliez & Stavritsch, 1922), and in order to determine the stability of this compound a sample of asparagine was heated with a known excess of formaldehyde at pH 1 and 100° for 1 hr. in a sealed glass tube. The tube was broken and the contents distilled in 0.3M-H₃PO₄. If 1 mol. of formaldehyde is equivalent to 1 mol. of asparagine, then the amount of combined formaldehyde, stable to distillation, was equivalent to 116.5% of the asparagine taken.

Table 3. The R_F values of asparagine and 6-hydroxytetrahydropyrimidine-4-carboxylic acid in phenol and *s*-collidine

Solvent	R_F values	
	Phenol-0.3% NH ₃	<i>s</i> -collidine
Asparagine	0.4	0.2
6-Hydroxytetrahydropyrimidine-4-carboxylic acid	0.91	0.45

The R_F values (Consden *et al.* 1944) obtained on paper chromatograms are summarized in Table 3. Asparagine gave a brown, and 6-hydroxytetrahydropyrimidine-4-carboxylic acid gave a yellowish brown spot with ninhydrin; 6-hydroxytetrahydropyrimidine-4-carboxylic acid was partly stable to hydrolysis at the boiling point for 4 hr. or at 38° for 22 days in 6N-hydrochloric acid. When this

hydrolyzed material was developed in phenol over ammonia, a purple spot of aspartic acid was obtained, together with a tailing yellowish brown spot of 6-hydroxytetrahydropyrimidine-4-carboxylic acid. When hydrolyzed in the presence of protein for 1 hr. in boiling 6N-hydrochloric acid, the substance is completely decomposed to aspartic acid.

Table 4. Free amino N of proteins after reaction with nitrous acid

(Free amino N as percentage of total nitrogen, after reaction for 1 hr. at 22° in 2N-HCl; all values are mean of two determinations.)

	Blacked-out chamber	In light
Carboxyhaemoglobin	5.55	6.59
Insulin (ash-free)	5.65	5.88
Edestin	2.12	2.16

The effect of Plimmer reagent on proteins

In order to determine the specific nature of the action of nitrous acid in 2N-hydrochloric acid on carboxyhaemoglobin, it was necessary to know the total free amino N of this protein; this was determined in the Van Slyke manometric apparatus, and also for insulin and edestin. The results are given in Table 4 which shows also the effect of blacking out the reaction chamber. A 40-W. tungsten filament lamp was placed near the reaction chamber of the manometric apparatus when carrying out determinations in the light. The results obtained for carboxyhaemoglobin confirm the findings of Fraenkel-Conrat (1943) that unduly high values are obtained unless the chamber is blacked out. However, for insulin and edestin the effect is slight.

Fraenkel-Conrat *et al.* (1945*a*) stated that treatment of proteins with sodium nitrite and hydrochloric acid would yield nitrogen equivalent to their primary amino, amide and three quarters of their arginine, together with small amounts from unspecified sources. However, as it has already been shown, it was impossible to remove any of the amide N from carboxyhaemoglobin under these conditions. The value obtained as percentage of total protein N was 10.52%. The theoretical value for the sum of free amino N (5.55%) and guanidino N (calculated from arginine N as 7.2% of total protein N (Macpherson, 1946)), was 10.95%.

DISCUSSION

The action of formaldehyde on the disulphide S of wool has been studied in previous papers (Bowes & Pleass, 1939*a, b*; Middlebrook & Phillips, 1942, 1947). Wormell & Kaye (1945) showed that under acid conditions and in the presence of salts at 35° formaldehyde would combine with the amide groups

of proteins. Fraenkel-Conrat *et al.* (1945*b*) also showed that this reaction would take place at elevated temperatures and higher pH values. If hair fibres are first boiled in formaldehyde at pH 1, they become resistant to supercontraction in boiling bisulphite solution, and this also applies to hair fibres which have been deaminated before the formaldehyde treatment. On deamidation, however, the fibres supercontract, but, as has been shown, only a fraction of the amide groups are removed, and these probably consist of all the glutamine but none of the asparagine amide group. It is, therefore, concluded that the resistance to supercontraction is due to cross-linkage formation between glutamine amide groups and others such as the guanidine groups of arginine (Fraenkel-Conrat & Olcott, 1946). These linkages are stable to boiling bisulphite solution, but can be completely removed by boiling the fibres in dilute phosphoric acid (see Table 1). The behaviour of fibres treated with formaldehyde at pH 10 can be explained by the formation of lanthionine cross linkages by the action of the alkaline buffer on the cystine cross linkages. As casein can combine with formaldehyde to produce linkages which are stable to boiling bisulphite solution, one formaldehyde mol. being equivalent to one amide group, it is unlikely that cross linkages are produced between two amide groups. Although distillation in dilute phosphoric acid removes the formaldehyde cross linkages, some of the formaldehyde still remains in combination with the protein. It has not been found possible to remove this formaldehyde, and estimate it satisfactorily, except by difference. The amount of irreversibly combined formaldehyde tends to a maximum at 4% formaldehyde concentration (Fig. 1), and if the minimal values of irreversibly combined formaldehyde between pH 1 and 2 (Fig. 2) are compared with the aspartic acid contents of the proteins (Table 2), then 1 mol. of formaldehyde is found to be equivalent to 1 mol. of aspartic acid. From these results, it is suggested that all the aspartic acid is in the form of asparagine (cf. Damodaran, 1932; Chibnall, 1942), which combines with formaldehyde in acid, at elevated temperatures, to form 6-hydroxytetrahydropyrimidine-4-carboxylic acid residues, stable to acid hydrolysis (Cherbuliez & Stavritch, 1922; Levy & Silberman, 1937). A sample of the free acid was prepared, and found to be stable to distillation in dilute phosphoric acid. Although *N*-substituted asparagine would combine with formaldehyde, ring closure apparently did not

occur, for this material was completely decomposed on distillation in dilute phosphoric acid.

Fully methylated wool combined irreversibly with less formaldehyde at pH 1 than untreated wool. This may be due to methylation of the asparagine amide groups.

Deamidation by the Plimmer (1925) technique removed only a part of the amide nitrogen and the amount of irreversibly combined formaldehyde was approximately unchanged. Of the proteins which were deamidated by this technique, the residual amide was approximately equivalent to the aspartic acid content. It is, therefore, probable that deamidation by the Plimmer technique only removes the glutamine amide nitrogen from proteins. It is significant that no deamidation took place in the case of carboxyhaemoglobin, where, if the aspartic acid content is taken to be 10.3% (Foster, 1945), all the amide nitrogen could be in the form of asparagine. Fraenkel-Conrat *et al.* (1945*b*) refer to the difficulty of removing all the amide nitrogen, and their results could be explained in this way. If confirmed, this might be used as a method to estimate the distribution of amide nitrogen in proteins between glutamine and asparagine. It should be noted that in all the proteins examined there is sufficient amide nitrogen to account for all the aspartic acid as asparagine. The free carboxyl groups in these proteins may arise from the glutamic acid residues and terminal amino-acids.

SUMMARY

1. In acid conditions at 100° proteins combine with formaldehyde, some of which cannot be removed by distillation in acid.
2. In the proteins examined, probably all the aspartic acid is in the form of asparagine residues, and the glutamic acid is in the form of glutamine and glutamic acid residues.
3. Formaldehyde forms cross linkages between the glutamic amide groups and other groups, possibly the guanidino groups of arginine. These cross linkages can be broken down by distillation in dilute phosphoric acid.
4. Formaldehyde probably combines with the asparagine amide groups to form 6-hydroxytetrahydropyrimidine-4-carboxylic acid, which is stable to boiling dilute phosphoric acid.

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Studies on the Fermentation of Ceylon Tea

8. FURTHER OBSERVATIONS ON THE RELATIONSHIP OF TEA FERMENTATION TO NORMAL RESPIRATION

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The original hypothesis of Roberts that all normal respiration ceases when tea leaf is rolled in the process of manufacture, and that the carbon dioxide observed during fermentation is due to a secondary carbohydrate oxidation by the o-quinones, has been further amplified in his two later communications (Roberts, 1941, 1943). This hypothesis, however, is in conflict with the finding in this laboratory

(Sreerangachar, 1941) that when the leaf is mechanically damaged, as in tea rolling, a certain amount of normal respiration still persists, thus accounting for the carbon dioxide produced by the fermenting leaf. This difference of opinion makes a critical examination of the evidence presented on both sides desirable, especially since these investigations, seriously interrupted during the war, can now be continued.

The problem centres round two main issues. First, is there any residual normal respiration in the

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