Tests with the anaerobes were conducted in Bacto-Anaerobe Medium with Dextrose. Cotton-stoppered tubes were filled with 9 cc. of the medium and placed in a boiling water-bath for twenty minutes and allowed to cool without agitation. To a series of 8 tubes the antibacterial solution was added in amounts to give 0.9, 0.7, 0.5, 0.25, 0.09, 0.07, 0.05 and 0.025 mg. per cc. of medium when all tubes received 1 cc. of a 1:1000 dilution of the organism to be tested in the anaerobe medium.

Bactericidal action was determined by removing 0.2 cc. of the medium from all tubes showing no growth after twenty hours of incubation and adding this to tubes containing 10 cc. of freshly heated and cooled Anaerobe Medium.

Discussion

The newly isolated compound from *Arctium minus* has a relatively low order of antibacterial activity. It is of interest, however, because of its presence in such relatively large quantities in burdock leaves and also from the standpoint of discovering new antibiotic types, a study of which may give evidence of their mode of action. The antibacterial activity is destroyed by treatment with cysteine or N-acetylcysteine but not with S-methylcysteine,[8] and in this respect resembles the behavior of certain other antibacterial agents.[8,9] It is also of interest to observe the frequency of unsaturated lactone structures among antibiotics, notably in anemonin,[10] patulin,[11] penicillic acid,[12] and now apparently in I.

Summary

A new antibacterial agent has been isolated from the leaves of *Arctium minus*. It has a relatively low order of activity against Gram-positive bacteria and is inactive against the Gram-negative group. The compound appears to be a lactone of empirical formula $C_{15}H_{20}O_5$.

(8) Cavallito and Bailey, *Science*, **100**, 390 (1944).

(9) Cavallito and Bailey, THIS JOURNAL, **66**, 1950 (1944); Cavallito, Buck and Suter, *ibid.*, **66**, 1952 (1944).

(10) Asahina and Fujita, *Acta Phytochim.* (Japan), **1**, 1 (1922).

(11) Raistrick, *et al.*, *Lancet*, **245** (2), 625 (1943).

(12) Birkinshaw, Oxford and Raistrick, *Biochem. J.*, **30**, 394 (1936).

RENSSELAER, N. Y. RECEIVED MARCH 22, 1945

[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

The Reaction of Formaldehyde with Proteins

BY HEINZ FRAENKEL-CONRAT, MITZI COOPER AND HAROLD S. OLCOTT

The reaction of formaldehyde with proteins is of importance in a number of fields, some of which are the tanning of leather, the hardening of tissues, fibers and plastics, and the preparation of toxoids. Much of the extensive earlier literature on the mode of interaction is cited in recent publications.[2-11]

The confusion existing in this field is emphasized by the fact that, in the four most recent papers, the binding of aldehyde by proteins in acid or neutral solution is attributed to the following groups: (1) all basic and phenolic and aliphatic hydroxyl[6b]; (2) imidazole, possibly amide and peptide, but not primary amino[9]; (3) primary amino and peptide[10]; and (4) primary amide and basic groups.[11] In addition, formaldehyde has been shown to react under certain conditions with the thiol,[12] indole,[13] guanidyl,[3] and disulfide groups.[5]

The present investigation was undertaken when it was observed that gliadin and wheat gluten bound more aldehyde than did other proteins, after treatment with 4% formaldehyde solution at 70° and at *p*H 3 to 7.[14] It was possible to demonstrate that the primary amide as well as the amino groups of proteins bind aldehyde under these conditions. On the other hand, the secondary amides of the peptide chain were found not to react appreciably with formaldehyde. These conclusions were derived from experiments with a series of proteins and protein derivatives, and with synthetic polypeptides and simple model substances.

Experimental

Analytical Methods.—Combined formaldehyde was determined by a method involving hydrolysis and distillation,[15] followed by precipitation of the aldehyde in the distillate with dimethyldihydroresorcinol (dimedon).[16] 100 or 200 mg. of the washed and dried protein derivative and 50 ml. of *N* sulfuric acid were placed in a 1000-ml. round-bottom flask, which was connected to a condenser fitted with a trap and an outlet tube dipping into a mixture of 50 ml. of 0.214% dimedon solution and 75 ml. of *p*H 4.6 acetate buffer (1 part *N* hydrochloric acid and 2 parts *N* sodium acetate). Distillation was continued until

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Theis and Ottens, *J. Am. Leather Chem. Assoc.*, **35**, 330 (1940).

(3) Highberger and Salcedo, *ibid.*, **35**, 11 (1940); **36**, 271 (1941).

(4) Brother and McKinney, *Ind. Eng. Chem.*, **30**, 1236 (1938).

(5) Middlebrook and Phillips, *Biochem. J.*, **36**, 294 (1942).

(6) (a) Carpenter and Lovelace, *Ind. Eng. Chem.* **34**, 759 (1942); (b) **36**, 680 (1944).

(7) Hegman, *J. Am. Leather Chem. Assoc.*, **37**, 276 (1942).

(8) Gustavson, *Kolloid Z.*, **103**, 43 (1943).

(9) (a) Theis, *J. Biol. Chem.*, **154**, 87 (1944); (b) Theis and Lams, *ibid.*, **154**, 99 (1944).

(10) Nitschmann and Hadorn, *Helv. Chim. Acta*, **27**, 299 (1944).

(11) Wormell and Kaye, *Nature*, **153**, 525 (1944).

(12) Ratner and Clarke, THIS JOURNAL, **59**, 200 (1937).

(13) Ross and Stanley, *J. Gen. Physiol.*, **22**, 165 (1938).

(14) The use of elevated temperature for the protein-formaldehyde reaction has been suggested by Ferretti (French Patent 853,123; March 11, 1940), Middlebrook and Phillips,[5] and others.

(15) Highberger and Retzsch, *J. Am. Leather Chem. Assoc.*, **33**, 341 (1939). The modification proposed by Nitschmann (*Helv. Chim. Acta*, **24**, 237 (1941)) was not found suitable for maximal recovery of the aldehyde bound by the casein at 70°, in agreement with a later paper from the same laboratory (Nitschmann, Hadorn and Lauener, *Helv. Chim. Acta*, **26**, 1069 (1943)).

(16) Yoe and Reid, *Ind. Eng. Chem., Anal. Ed.*, **13**, 238 (1941).

sulfuric acid fumes began to fill the distillation flask. Any precipitate adhering to the outlet tube was washed into the receiver, the content of which was filtered after four to forty-eight hours. The precipitate was washed, dried for sixteen hours at 50° or for three hours at 70°, and weighed.[17]

Free amino groups were determined by a fifteen-minute reaction period in the Van Slyke manometric apparatus[18] with a blacked-out chamber.[19] Total basic groups of untreated proteins were determined by the recently developed dye method.[20] The primary amide groups of untreated proteins were determined by partial hydrolysis (forty minutes at 120° in 1.2 *N* sulfuric acid), followed by distillation of the ammonia. Neither of the latter two methods permitted a differentiation between free and aldehyde-reacted basic or amide groups because of the lability of the N-methylol bond. The method of determining the sum of primary amide, amino and guanidyl groups by the Plimmer technique[21,22] proved applicable for this purpose.

Method of Preparation of Formaldehyde Derivatives.—In most experiments 1 g. of protein or polypeptide[23] was mixed with, or dissolved in, 8 ml. of water.[24] To this was added 1 ml. of buffer and 1 ml. of 37–38% formaldehyde solution. 3 *M* acetate buffer of *p*H 3.5 was used in the acid region and 3.4 *M* phosphate buffer of *p*H 8.2 in the neutral region. The *p*H values measured at the end of the reaction period were usually 3.8 and 6.8, respectively. The samples, in stoppered flasks, were kept in an oven at 70° for four days and shaken at intervals. After this treatment all of the proteins studied, with the exception of gelatin,[25] were sufficiently insoluble in water to permit centrifugation and thorough washing. They were usually washed ten times, each time with 40 ml. of water, and then dehydrated with alcohol and ether. An alternate method of washing, applicable also to soluble aldehyde-treated proteins, consisted in dialyzing the solutions or suspensions for three days against running tap water and one day against distilled water. The aldehyde contents of the final products were found to vary by no more than 10% with the different techniques of isolation.

Effect of Experimental Conditions on Extent of Reaction of Proteins with Formaldehyde.—A reaction period of four days at 70° resulted in products containing maximal amounts of formaldehyde; 50% of the final amount of aldehyde was bound within eight hours, and 90% within twenty-four hours.

The effect of the *p*H of the reaction mixture on the amounts of formaldehyde bound depended upon the nature of the protein groups involved in the reaction. Lysozyme bound about 37% more at *p*H 6.8 than at *p*H 3.8; egg white protein, 18% more; gluten, 10% more; zein, the same amount at both *p*H levels; and polyglutamine, 38% less at the higher *p*H.

The aldehyde concentration and the reaction temperature were found to influence considerably the maximal amount of formaldehyde that could be introduced. Thus gluten bound about 2% of its weight of formaldehyde when treated either at room temperature with 3.8% formaldehyde solution or at 70° with 0.75% formaldehyde. The use of 18% aldehyde solution at 70° (*p*H 3.8) led to the introduction of 7% of the reagent, as compared to the 6% introduced from 3.8% aldehyde solution.

Stability of Bound Formaldehyde.—The formaldehyde retained by the proteins after the usual washing procedure was comparatively stable during further prolonged contact with water at room temperature. After seven days of dialysis against running tap water, the aldehyde contents of gluten and gliadin were about 10% lower than after three days of dialysis. Steam distillation caused the release of most of the bound aldehyde. Holding the dry materials at 100° for seven days reduced the aldehyde contents by about 60 to 70%; at 150°, by 85% in three days.

Dialysis of suspensions of washed aldehyde-treated gluten against 1% sodium sulfite caused the loss of 50 to 60% of the aldehyde in three days; the residual aldehyde was stable to further treatment with sulfite. The aldehyde bound by polyglutamine was split off in sodium sulfite, but not in bisulfite solution. Thus exhaustive "washings" of aldehyde-treated proteins with sulfite solution cannot be regarded as a technique suitable for the removal of the unbound aldehyde alone, for which it has been advocated.[3,7]

Preparation of Polyglutamic Acid, Polyglutamic Methyl Ester, and Polyglutamine.—The polypeptide of *d*(−)-glutamic acid was prepared by the method of Bovarnick[26] with the coöperation of J. C. Lewis of this Laboratory. This material is elaborated by a particular strain of *B. subtilis*,[27] and is isolated by the precipitation of its copper salt from the medium. After the removal of foreign material and inorganic ions as described by Bovarnick,[26] the residual solution was dried from the frozen state.

Anal. Calcd. for $(C_5H_7O_3N)_x$: N, 10.85; amino N (after hydrolysis), 10.85; glutamic acid (after hydrolysis), 114; equiv. wt., 129. Found: N, 10.6; amino N (after hydrolysis), 10.5; glutamic acid,[28] (after hydrolysis) 110; equiv. wt., 130.5.

In order to prepare the methyl ester, the acid polypeptide was esterified with diazomethane in ether or, preferably, by the method of Freudenberg and Jacob.[29] A dialyzed aqueous solution of the ester had a *p*H of 6.6, in contrast to the original polypeptide which, in aqueous solution, is at *p*H 2.8. Titration with 0.1 *N* sodium hydroxide and phenolphthalein indicated that over 97% of the carboxyl groups had been esterified. The solution was dried by lyophilization.

Anal. Calcd.: N, 9.8; methoxyl, 21.6. Found: N, 9.7; methoxyl, 20.8.

The polyamide was obtained as follows: 1 g. of polyglutamic acid methyl ester was suspended in approximately 50 ml. of liquid ammonia in a Dewar flask. A small amount of ferric chloride was added as catalyst. After

(17) The soluble formaldehyde reaction products of simple amides and diketopiperazines were found to be stable under the conditions of dimedon precipitation. It was thus possible to determine the amount of formaldehyde bound by these compounds by comparing the free aldehyde, as determined in an aliquot by direct addition of dimedon and buffer, with the total aldehyde as determined in another aliquot by hydrolysis and distillation. The same technique could not be used with amines or amino acids, the formaldehyde reaction products of which appeared unstable in the presence of dimedon at *p*H 4.6. This was indicated by the finding that the losses in the primary amino nitrogen of these compounds due to reaction with formaldehyde were greatly in excess of the amounts of aldehyde which they retained in the presence of dimedon. A similar phenomenon was recently described by Neuberger, *Biochem. J.*, **38**, 309 (1944).

(18) Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

(19) Fraenkel-Conrat, *ibid.*, **148**, 453 (1943).

(20) Fraenkel-Conrat and Cooper, *ibid.*, **154**, 239 (1944).

(21) Plimmer (*J. Chem. Soc.*, **127**, 265 (1925)) has shown that these groups liberate nitrogen quantitatively upon treatment with nitrous acid in the presence of mineral acid. This method has recently been applied to proteins.[22]

(22) Fraenkel-Conrat, Cooper and Olcott, THIS JOURNAL, **67**, 314 (1945).

(23) The proteins and the nylon and polyglycine preparations were the same as those used in previous studies.[20,22] The preparation of polyglutamic acid and polyglutamine is described below.

(24) In the case of gliadin, 50% ethanol was a more suitable reaction medium than water.

(25) Gelatin set to a gel at 70° after a few hours of aldehyde treatment. After twenty-four hours the reaction mixture had liquefied and the protein remained soluble in cold water even after dialysis. The aldehyde content did not increase appreciably during this transformation.

(26) Bovarnick, *J. Biol. Chem.*, **145**, 415 (1942).

(27) The organism used was No. B-571 of the Northern Regional Research Laboratory stock culture collection.

(28) Determined by the method described by Olcott, *J. Biol. Chem.*, **153**, 71 (1944).

(29) Freudenberg and Jacob, *Ber.*, **74B**, 1001 (1941). The application of this method to the esterification of proteins will be described in a future publication.

two days, the excess ammonia had evaporated. The product was taken up in water, dialyzed and lyophilized. Analyses indicated that about 80% of the ester groups had been transformed to amide groups.

Anal. Calcd. for mixed amide ester $((C_5H_8O_2N_2)_4\text{-}(C_6H_9O_3N))_x$: N, 19.2, amide N, 8.6. Found: N, 18.9, amide N, 8.9.

Results

Protein Groups Responsible for the Binding of Formaldehyde.[30]—When proteins were treated with 4% formaldehyde at 70° and at *p*H 3.5 to 4.0, the resultant products, after thorough washing, contained amounts of formaldehyde that ranged from 7% for gliadin to 0.7% for silk fibroin. The amino nitrogen contents of the treated proteins were reduced to 10 to 20% of those of the starting materials.[31] Yet the amounts of aldehyde bound by most of the proteins were greatly in excess of those equivalent to the amino or even to the total basic groups. However, it was possible to demonstrate a correlation between the sum of the basic and the amide groups of proteins and their capacity to bind formaldehyde (Table I). These findings were regarded as suggestive evidence that the primary amide groups, together with the basic groups,[32] are responsible for a great part of the aldehyde bound by proteins under the conditions used.

This conclusion was supported by a comparison of the aldehyde-binding capacities of proteins or polypeptides modified or prepared in such a manner as to contain maximal or minimal numbers of the reactive groups (Table I). Thus proteins, the amide and basic groups of which had been decreased by treatment with nitrous acid in 2 *N* hydrochloric acid[21] or through reaction with phenyl isocyanate,[22] bound considerably less aldehyde than did the original proteins. Polypeptides of glutamic acid or glycine and the polyamide, nylon, also bound very little aldehyde, which indicates that the carboxyl, and peptide or secondary amide groups do not react to an appreciable extent. On the other hand, a polypeptide containing many amide groups (polyglutamine) combined with more formaldehyde than did any other macromolecular material.

A direct demonstration of the loss in free primary amide groups in proteins through combination with formaldehyde was possible by Plimmer's technique.[21,22] The decreases which were observed after aldehyde treatment of proteins rich in amide groups (Table II) indicate clearly that these groups participate in the reaction.[33]

TABLE I

CORRELATION OF THE AMOUNT OF FORMALDEHYDE BOUND AT *p*H 3.5 TO 4 AND 70° BY PROTEINS, DERIVED PROTEINS AND POLYPEPTIDES WITH THEIR REACTIVE GROUPS[a]

	Groups per 10^4 g.				Moles of aldehyde bound per 10^4 g.
	Primary amino[b]	Total basic	Primary amide	Basic + amide	
Polyglutamine	2.4	2.4	60	62.4	47
Gliadin	1.3	4.3	30	34.3	23
Gluten	1.3	5.3	21	26.3	20
Lysozyme	3.6	12.5	10	22.5	13
Zein	1.2	1.9	18	19.9	15
Casein	4.8	6.8	10	16.8	12
Hoof powder	4.5	8.8	7.6	16.3	12
Egg white proteins	4.5	8.2	7.1	15.3	11
Wool keratin	1.8	8.2	7.9	16.1	11
Egg albumin (cryst.)	4.3	8.8	7.8	16.6	9
Feather keratin	1.5	5.1	7.4	12.5	8
Zein, partly deamidated[c]	1.0	0.3	11.3	11.6	8
Gelatin	3.5	6.0	2.9	8.9	6
Casein, partly deamidated[c]	0.7	2.2	6.6	8.8	6
Gliadin, phenyl isocyanate treated[d]	0.0	1.1	7	8	9
Egg white, phenyl isocyanate treated[d]	0.0	0.3	7	7	4
Gluten, partly deamidated[c]	1.7	1.1	3.0	4.1	4
Gluten, phenyl isocyanate treated[d]	0.2	0	4	4	5
Polyglycine[d]	2.4	2.8	0	2.8	3.0
Polyglutamic acid	1.9	1.9	0	1.9	2.6[e]
Silk fibroin	0.7	1.3	0.4	1.7	2.3
Nylon	0.1	0.6	0	0.6	0.3

[a] Treated with 3.75% formaldehyde solution for four days. The lysozyme, crystalline egg albumin, and silk fibroin were kindly furnished by H. L. Fevold, E. F. Lindquist, and M. Bergmann, respectively. For method of preparation of polyglutamic acid and polyglutamine, see text. [b] After aldehyde treatment, the amino nitrogen of all proteins was reduced by 80 to 90%. [c] By an application of the Plimmer technique[21] to a preparative scale, which did not lead to as complete deamidation as did the analytical procedure. [d] The preparations were obtained as previously described.[22] The amide nitrogen of these samples was determined by the Plimmer technique,[21] since methods based on partial hydrolysis cannot be used with phenyl isocyanate-treated proteins.[22] [e] This reaction took place at *p*H 6.8. When heated at *p*H 3.8, polyglutamic acid became dialyzable. Polyglutamine bound 31 moles of aldehyde at *p*H 6.7.

Discussion

The amounts of formaldehyde bound by a num-

(30) While the present paper was being prepared for publication, the preliminary note of Wormell and Kaye[11] came to our attention. Our data indicate that the reaction of amide groups with formaldehyde does not require the presence of mineral acid and saturated calcium chloride as used by Wormell and Kaye. Thus zein bound 4.5% of its weight of formaldehyde in acetate buffer at *p*H 3.8; 4.1% under the conditions used by these investigators.

(31) These results cannot be attributed to the insolubility of tanned proteins, since it was possible to obtain consistent values for insoluble products such as keratins and for denatured or derived proteins used as powders of varying particle size. The water-soluble aldehyde derivative of gelatin also had a low amino nitrogen content.

(32) No attempt was made to ascertain the extent of participation of the imidazole and guanidyl groups of the various proteins in the formaldehyde reaction. Several previous investigators have indicated that the former but not the latter groups are involved below *p*H 9. However, evidence that guanidyl groups in proteins bind formaldehyde even below *p*H 7.0 will be reported later.

(33) The reaction of simple amides with formaldehyde was studied in detail by Einhorn, *Ann.*, **343**, 207 (1905); **361**, 113 (1908); and more recently by Noyes and Forman, THIS JOURNAL, **55**, 3493 (1933). Under the conditions used by us with proteins, N-methylol acetamide was formed from acetamide and formaldehyde. Methylene diacetamide resulted from the decomposition of N-methylol acetamide at 150°. These compounds were found to yield 70 to 90% of their nitrogen on treating with nitrous acid in the presence of mineral acid. If it is assumed that part of the amido-methylol groups of proteins may also be hydrolyzed under these conditions, the extent of blocking of amide groups may be actually greater than indicated by the data in Table II.

TABLE II

EFFECT OF FORMALDEHYDE ON THE LABILITY OF THE AMIDE, AMINO AND GUANIDYL GROUPS OF PROTEINS TOWARD NITROUS ACID[a]

	Nitrogen liberated by treatment with nitrous + hydrochloric acid[a]		Nitrogen in starting material	
	Formaldehyde derivative, %	Starting material, %	Amide,[b] %	Amide + amino + guanidyl,[b] %
Gliadin	3.4	5.4	4.3	5.1
Zein	1.9	3.5	3.0	3.4
Polyglutamine	1.7	10.4[c]	8.9	9.2

[a] Reacted in volumetric Van Slyke apparatus for twenty-four hours at room temperature, in 2 *N* hydrochloric acid (*cf.* footnotes 21 and 22). [b] The amide and amino nitrogen values were determined; the guanidyl-nitrogen values were calculated from data in the literature. [c] Polyglutamic acid is partially hydrolyzed under these conditions. A similar lability of the peptide links of polyglutamine may explain the observed high value.

ber of proteins under controlled conditions, and released by acid hydrolysis, have been determined. While it is well recognized that the reactions involved are largely reversible and therefore dependent upon conditions of both treatment and washing, it is noteworthy that the data obtained agree in general with comparable data in the literature.

The identity of the protein groups involved in this reaction has been more frequently the object of conjecture than of exhaustive study. Thus, on the basis of titration curves, Theis recently concluded[9] that the primary amino groups of proteins react only above *p*H 9.5. This conclusion was not supported by amino nitrogen determinations. Nitschmann and Hadorn,[10] however, observed marked decreases in the amino nitrogen of casein after formaldehyde treatment at *p*H 5 to 6. The present study has revealed consistent losses of 80 to 90% of the primary amino nitrogen of proteins upon aldehyde treatment in the range of *p*H 3.5 to 7.0.

It has recently been assumed by Carpenter and Lovelace[6a,b] that, in addition to all basic groups, aliphatic hydroxyl groups bind formaldehyde through acetal linkages, and tyrosine binds formaldehyde through addition in the 2,6 positions. These claims were based on the observation that the amount of aldehyde maximally introduced into one protein, casein, at room temperature and at high aldehyde concentration agreed with calculations based on such assumptions. To obtain similar agreement between "expectation" and analytical results in the case of deaminated casein, the further assumption was made that the nitrous acid caused 2,6 substitution of all phenolic groups present, thus preventing the tyrosine in deaminated casein from binding formaldehyde. In contrast to these assumptions, Ross and Stanley[13] and Kassanis and Kleczkowski[34] found that the indole ring of tryptophan, but not the phenolic ring of tyrosine, loses its ability to react with Folin's phenol reagent after aldehyde treatment. While the present investigation was not primarily concerned with this problem, the fact that silk fibroin binds very little formaldehyde also casts doubt upon the reactivity of either aliphatic[35] or phenolic hydroxyl groups, since fibroin is rich both in serine and tyrosine. The failure of an attempt to introduce formaldehyde into *p*-cresol under the conditions used in this study is further evidence that the phenolic ring of proteins is not reactive.[36]

Reaction of the amide groups and of the peptide nitrogen of proteins with formaldehyde has often been hypothesized.[6a,8,9,37,38] That the primary amide groups actually participate in the reaction in acid solution has recently been shown by Wormell and Kaye[11] and by the present study. The relative reactivity of polyglutamine at *p*H 3.5 and 6.8 indicates that amide groups react with formaldehyde more extensively in acid than in neutral solution, in agreement with the conclusions of Wormell and Kaye.[11] On the other hand, the reactivity of basic groups is known to be favored by decreasing acidity of the solution.[2,7,8,9] Thus the ratio of amide to basic groups determines the effect of *p*H on the amount of aldehyde bound by various proteins.

Carpenter and Lovelace[6] concluded that amide groups do not bind formaldehyde, from their observation[39a,b] that asparagine bound no more formaldehyde than did aspartic or glutamic acid. It appears doubtful that asparagine is a suitable model system for the study of the reaction of protein amide groups, since it contains a reactive amino group in proximity to the amide group. Levy and Silberman[40] had previously suggested an interpretation of the asparagine–formaldehyde reaction, in which one molecule of formaldehyde closed a ring involving both the amino and the amide groups. This reaction mechanism may explain the equal amounts of formaldehyde bound by asparagine and aspartic acid That the two reaction products differ in the mode of linkage of formaldehyde is evident from their stability in the presence of dimedon. Carpenter and Lovelace, reporting data obtained by this technique only in their earlier paper on asparagine,[39a] found this substance to bind one equivalent of formaldehyde in a stable manner in alkaline solution. We

(34) Kassanis and Kleczkowski, *Biochem. J.*, **38**, 20 (1944).

(35) Under the conditions described in this paper polyvinyl alcohol bound 2% formaldehyde at *p*H 3.0, which is equivalent to only 5% of the available hydroxyl groups. At *p*H 7.0, the amount of formaldehyde bound was negligible.

(36) 1% solutions of *p*-cresol were treated with 4% formaldehyde both at *p*H 3.8 and 6.7. There was no aldehyde bound after four days at 70°. The added formaldehyde was quantitatively recoverable either by distillation or by direct precipitation with dimedon. The chromogenic value (by the Folin phenol test) of the aldehyde-treated sample was the same as that of a similarly treated control solution.

(37) Holland, *J. Int. Soc. Leather Trades Chem.*, **23**, 215 (1939).

(38) Waldschmidt-Leitz, *Die Chemie*, **55**, 62 (1942).

(39) Carpenter and Lovelace, (a) THIS JOURNAL, **64**, 2899 (1942); (b) **65**, 1161 (1943).

(40) Levy and Silberman, *J. Biol Chem.*, **118**, 723 (1937).

found that the stable addition product is formed also at *p*H 3.8. However, when the dimedon technique was applied to a comparable reaction mixture of aspartic acid with formaldehyde, the product was labile, as were most other amino-methylol compounds investigated.[17] The experiments of Carpenter and Lovelace thus do not constitute evidence against the reaction of protein amide groups with formaldehyde.

The ability of glycine anhydride to bind formaldehyde[41] is frequently quoted in support of the assumption that a similar reaction occurs with the amide nitrogen of the peptide chain. That diketopiperazines are more active than are chain peptides is well known and has been supported by recent evidence.[22] The difference between the two types of —CO—NH— linkage is evident also from the results of the present study in which it is shown that nylon, straight-chain peptides and silk fibroin bind little formaldehyde under conditions which favor the reaction of glycine anhydride with formaldehyde.[42] The contrast between the inactivity of the chain peptide link and the reactivity of the primary amide group is clearly illustrated by the behavior of polyglutamic acid and polyglutamine upon treatment with aldehyde. The former bound less than 1 aldehyde molecule for 100 glutamic acid residues; the latter bound as much as 88 aldehyde molecules for 100 amide residues.

Nitschmann and Hadorn[10] recently restated the theory that the peptide nitrogen of casein is mainly responsible for the amount of fixed formaldehyde, in excess of that bound to lysine. They arrived at this conclusion by the elimination of other protein groups, with the surprising omission of the primary amide groups from their discussion. In support of their conclusion, they mention the reactivity of glycine anhydride. Further, these authors refer to an earlier study of the reaction rate that indicated two separate reaction types. They now interpret the rapid one as involving the amino groups. The slow reaction was greatly dependent upon aldehyde concentration and appeared to continue indefinitely at room temperature. This led Nitschmann and Hadorn to conclude that it must be due to groups present in relatively large numbers in proteins, namely, the peptide groups.

In the present study the reaction of primary amide groups with formaldehyde was found to be slow and greatly dependent upon aldehyde concentration and reaction temperature. Nitschmann and Hadorn's data can thus also be interpreted as indicating the reaction of the amide groups. The difference between the two studies lies mainly in the reaction temperature. At the elevated temperatures that we used, equilibrium was reached within a reasonable time. The amount of aldehyde bound at this point did not exceed the number of reactive side chains, and it was therefore not necessary to hypothesize that the peptide groups were involved. This factor, as well as the use of many different proteins, facilitated the recognition that the primary amide groups and not the peptide bonds are responsible for the slow fixation of formaldehyde.

The results do not exclude the possibility that a few particularly reactive peptide bonds participate in the reaction. Nor is the possibility of a secondary condensation of the amino-methylol groups with the peptide chain, as suggested by Nitschmann and Hadorn[10] disproved. However, this condensation reaction may also occur with other reactive hydrogen atoms.

Acknowledgment.—We thank G. H. Brother of this Laboratory for his continued interest and encouragement, and J. C. Lewis for his helpful cooperation in the preparation of polyglutamic acid.

Summary

In 4% solution, at *p*H 3 to 7 and 70°, formaldehyde combined with both the primary amino and the primary amide groups of proteins. In contrast to interpretations of other investigators, the secondary amide linkages of the peptide chain, and the phenolic groups, were found not to bind appreciable amounts of formaldehyde.

These findings were confirmed with protein derivatives and synthetic polypeptides containing a maximal or minimal number of reactive groups.

The preparation of a polypeptide rich in primary amide groups from polyglutamic acid is described. This material bound more formaldehyde than did any of the proteins investigated.

RECEIVED NOVEMBER 16, 1944

(41) Cherbuliez and Feer, *Helv. Chim. Acta*, **5**, 678 (1922); Bergmann, Jacobson and Schotte, *Z. physiol. Chem.*, **131**, 18 (1923).

(42) The formation of N,N'-dimethylol diketopiperazine from glycine anhydride and formaldehyde was found to occur under our experimental conditions. Alanine anhydride and higher molecular weight diketopiperazines appeared to react to a progressively decreasing extent.