Summary

New derivatives of 6-methoxy-8-aminoquinoline have been prepared. Most of these contain the dialkylaminoethyl side chain variously substituted. Two are substituted diquinolyl amines.

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

Reaction of Formaldehyde with Proteins. II. Participation of the Guanidyl Groups and Evidence of Crosslinking

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The reaction of formaldehyde with amino acids and proteins has received renewed attention and both scientific and practical application in recent years. In contrast to older aldehyde tanning procedures, elevated temperatures and acid reaction media have been found suitable for the hardening of casein fibers.[2] Such conditions have been shown to favor the reaction of the primary amide groups of proteins with formaldehyde.[3] It has long been recognized that primary amino groups are involved in this reaction. With the use of protamine as a model substance, evidence has now been obtained that guanidyl groups bind formaldehyde in neutral and acid solutions at 70°, a reaction which at room temperature has been described as proceeding readily only in the alkaline *p*H range.[4]

The tanning action of formaldehyde on proteins has never been clearly understood. It was at one time believed that it might be due to the transformation of the original amino groups to Schiff bases ($-NH_2 + HCHO \rightarrow -N{=}CH_2 + H_2O$). More recent evidence[5] favors a simple addition reaction, leading to imino or imido methylol ($-NH-CH_2OH$) groups, but the marked decrease in hydrophilic tendency and increase in strength produced in proteins through tannage cannot readily be explained on the basis of either of these reactions. The hypothesis that has received most general acceptance is that formaldehyde sets up crosslinks by secondary condensation of the methylol groups with other reactive hydrogen atoms, similar to that which occurs in the Mannich reaction ($-NH-CH_2OH + HR \rightarrow -NH-CH_2-R + H_2O$).[6] It was not until recently that chemical evidence for a condensation reaction was obtained by Nitschmann and Hadorn,[7] who showed that there was a loss in weight, presumably water, accompanying the addition of formaldehyde to the casein molecule. While we were able to confirm this observation, it is recognized that the formation of Schiff bases would also lead to a loss of water. Further, the condensation reaction might involve the amino and peptide groups of the same lysine residue and thus might not lead to crosslinking of adjacent peptide chains.

It is apparent that intermolecular crosslinking through formaldehyde causes an increase in the average molecular weight of proteins. The decreased solubility of treated proteins may be attributed to this phenomenon. This insolubility on the other hand has probably discouraged quantitative investigations of the molecular weights of aldehyde derivatives of proteins. The protamine (salmine) used in the present study of the reactivity of guanidyl groups remained largely soluble when dilute solutions (1–3%) were treated with formaldehyde. In contrast to untreated protamine, the aldehyde derivative prepared at *p*H 6–7 was partially retained by cellulose bags upon dialysis. Osmotic pressure measurements showed that this material was of considerably greater average molecular weight than the untreated material. These observations constitute evidence for the formation of intermolecular crosslinks by formaldehyde under the experimental conditions used.

Experimental

Reaction of Protamine with Formaldehyde.—The conditions generally used were as follows: To 1 g. of protamine sulfate[8] dissolved in 80 ml. of warm water there was added 10 ml. of 3.4 *M* phosphate buffer (*p*H 7.6), or 3 *M* acetic acid, and 10 ml. of commercial 40% formaldehyde solution. The mixtures were held at 70° for four days; these conditions led to the establishment of equilibrium. The concentrations were not critical. Similar products were obtained with protamine concentrations up to 3% and with 16% formaldehyde.

The mixtures were found to be close to *p*H 6.5 or 2.8, respectively, at the end of the heating period. When the reaction proceeded in the absence of phosphate buffer, the solution dropped from neutrality to below *p*H 4. Small amounts of insoluble matter were filtered off before the reaction mixtures were analyzed. The approximate amount of formaldehyde bound by protamine at equilibrium was determined by measurements of the difference between the total and the free formaldehyde in the reaction mixture.[3] To this end an aliquot of the solution was hydrolyzed with sulfuric acid and the aldehyde distilled into

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

(2) Franz, Riederle, Fleischmann and Winkler, *J. prakt. Chem.*, **160**, 133 (1942).

(3) Fraenkel-Conrat, Cooper and Olcott, THIS JOURNAL, **67**, 950 (1945); Wormell and Kaye, *J. Soc. Chem. Ind.*, **64**, 75 (1945).

(4) Hegman, *J. Am. Leather Chem. Assoc.*, **37**, 276 (1942); Salcedo and Highberger, *ibid.*, **36**, 271 (1941).

(5) Levy and Silberman, *J. Biol. Chem.*, **118**, 783 (1937).

(6) (a) Gustavson, *Kolloid Z.*, **103**, 43 (1943); (b) Smith, Handler and Mrgudich, *J. Phys. Chem.*, **44**, 874 (1940).

(7) Nitschmann and Hadorn, *Helv. Chem. Acta*, **27**, 299 (1944).

(8) Approximately pure salmine sulfate, kindly supplied by Eli Lilly and Co.

dimedon solution, while another aliquot was added directly to the dimedon solution.

The isolation of the formaldehyde-treated protamine required techniques differing from those used with other proteins.[8] Thus its water solubility and low molecular weight precluded its quantitative separation from excess formaldehyde and buffer salts by means of washing or dialysis. Removal of free formaldehyde was achieved by repeated precipitation of the protamine derivative from concentrated aqueous solution with acetone. Phosphate buffer salts could largely be separated from the protamine derivative through their low solubility in 50% aqueous methanol. The removal of the phosphate leads to the separation of the protamine derivative as a solid rather than in an aqueous layer. After 4–6 cycles of solution and precipitation, the products were analyzed in aqueous solution. Kjeldahl nitrogen analyses usually indicated 50–90% recovery (Table I). Determinations of free aldehyde were carried out as a measure of the thoroughness of the washing procedure, and the small amounts present were subtracted from the total aldehyde found after hydrolysis. The aldehyde bound by the protamine, computed in this matter, was expressed in terms of moles per mole of nitrogen.

TABLE I

FORMALDEHYDE TREATMENT OF VARIOUS GUANIDINE DERIVATIVES[a]

Material	Initial *p*H	Nitrogen recovery, %	Solution of reaction product: Aldehyde per mole N, Bound Mole	Aldehyde per mole N, Total Mole	Plimmer N[b] per mole total N Mole
Protamine	7.6	89	0.38[d]	0.41	0.34
sulfate[c]	7.6	35[e]	.35[d]	.38	.12
	3.2	50	.18[d]	.21	.55
Arginine	7.6	62	.45[g]	.56	.55
hydrochloride[f]	3.2[h]	39	.38[g]	.41	.53
Methylguanidine	7.6	49	.59[i]	.69	.27
sulfate[f]	3.2	84	.66[i]	.67	.36
Guanidine	7.6	55	.84[k]	.98	.18
hydrochloride[f]	3.2	67	.87[k]	.99	.48

[a] The experiments listed were performed at 70°, with a reaction period of 4 days. More extensive treatment was no more effective. At room temperature, about half the amount of aldehyde was introduced into the simple guanidines in ten to twenty days, even less into protamine. [b] Nitrogen liberated by nitrous acid in hydrochloric acid in 24 hours at room temperature.[13] Values for untreated protamine, 0.61; arginine, 0.87; methylguanidine, 0.75; and guanidine, 0.90. [c] 1.0 g. of protamine treated with 100 ml. of 4% formaldehyde solution containing 10 ml. of 3.4 *M* *p*H 7.6 phosphate or 3 *M* acetic acid. At twice these concentrations of both reactants, as much as 0.45 mole of aldehyde was introduced at *p*H 7.6. The ratio of Plimmer N to total N in such preparations, isolated by dialysis, was only 0.09–0.14. When 16% formaldehyde was used, 0.43 mole of aldehyde was introduced, both at *p*H 3.2 and 7.6, and the Plimmer N was 25 and 11% of the total N, respectively. [d] These figures, as determined at equilibrium in the reaction mixtures, were 0.40, 0.37 and 0.21, respectively. [e] Isolated by dialysis. [f] 0.4 *M* solution containing 16% formaldehyde and 20% of the buffer solution. In more dilute solution, equilibrium was not reached in four days at 70°. [g] These figures, as determined at equilibrium in the reaction mixtures, were 0.46 and 0.36, respectively. [h] Secondary reactions were indicated by appearance of a brown color and development of a gas. [i] These figures, as determined at equilibrium in the reaction mixtures, were 0.66 and 0.64, respectively. [k] These figures, as determined at equilibrium in the reaction mixtures, were 0.93 and 0.97, respectively.

Occasionally dialysis was employed as a means of isolating the reaction products free from low-molecular weight contaminants. Untreated protamine, as well as protamine heated at neutrality in the absence of formaldehyde, and protamine-aldehyde derivatives prepared at *p*H 3, passed through the bags (Visking tubing, 30/32″ diam.) almost completely, *i. e.*, to about 95%, during dialysis against running tap water for three days. However, 20 to 50% of the aldehyde derivatives prepared at neutrality were retained under those conditions. The molar ratio of bound aldehyde to nitrogen in the dialyzed fractions was similar to that of products isolated by repeated precipitation (Table I). The dialyzed material contained both sulfate and phosphate ions. In order to obtain the derivative entirely in the form of the sulfate for analytical purposes, its solutions were half saturated with ammonium sulfate, adjusted to *p*H 4 with sulfuric acid, and dialyzed. The aldehyde derivative isolated in this manner contained 22.5% N and 1.9% sulfate S, compared to 24.6% N and 6.7% sulfate S for untreated protamine sulfate (both on dry basis).

Reaction of Arginine, Methylguanidine and Guanidine Salts with Formaldehyde.—To 0.01 mole of arginine hydrochloride, methylguanidine sulfate, or guanidine hydrochloride were added 10 ml. of 40% formaldehyde (0.13 mole), 5 ml. of 3 *M* acetic acid or 3.4 *M* phosphate buffer (*p*H 7.6), and distilled water to a total volume of 25 ml. Equilibrium was reached within four days at 70° in such reaction mixtures, but not in more dilute solutions. Arginine was found to turn brown and develop pressure when reacted in acid solution, but the other mixtures appeared unchanged.

The amount of aldehyde bound by the various samples was determined by the methods used with protamine. That bound at equilibrium was calculated from the difference between total and free aldehyde in aliquots of the reaction mixture. The arginine derivatives were freed from excess formaldehyde in the same manner as described for protamine. The guanidine derivatives were largely soluble in acetone and were therefore repeatedly redissolved in methanol and precipitated by ether. Prolonged repetition of this treatment led to a gradual loss of a fraction of the bound aldehyde, particularly in the case of unsubstituted guanidine. Usually 60–80% of the nitrogen was recovered in the final solutions.

The formaldehyde derivative of methylguanidine sulfate obtained in acid solution proved to be the most stable and the most easily purified product. Analyses for nitrogen, sulfate, and formaldehyde indicated ratios in good agreement with expectation for a neutral dimethylol methylguanidine sulfate. However, the percentages of the three components were somewhat higher than those calculated for this compound. The analyses indicate the loss of water through formation of methylene groups during the reaction or isolation of the material.

Anal. Calcd. for $(C_4H_{11}O_2N_3)_2 \cdot H_2SO_4$: N, 23.1; S, 8.8; HCHO, 33.0. Same minus $1H_2O$: N, 24.3; S, 9.3; HCHO, 34.7. Same minus 1.2 H_2O: N, 24.6; S, 9.4; HCHO, 35.1. Found: N, 24.6; S, 9.7; HCHO, 34.7.

Results

When protamine is treated with formaldehyde at neutrality and 70°, the reagent is introduced to an extent of 14–20% of the weight of the derivatives, depending upon concentration. These amounts exceed those which can be introduced into typical proteins.[8] The aldehyde bound corresponds to 0.29–0.42 mole per mole nitrogen. Since 89% of the nitrogen of salmine is arginine N,[9] this figure would be 0.22 if each guanidyl residue bound one mole of formaldehyde. It thus appears that as much as two equivalents of the aldehyde can be bound by each guanidyl group.

(9) Kossel, "The Protamines and Histones," Longmans, Green and Co., London, 1928, p. 85; Block and Bolling, *Arch. Biochem.*, **6**, 419 (1945).

Model experiments with various guanidines support this conclusion. Arginine and methyl guanidine bind up to two, and guanidine almost three, moles of formaldehyde under the same conditions. In the absence of buffer, or in the presence of acetic acid, maximal amounts of aldehyde are bound only if the reagent concentration is high (16%). Arginine shows signs of decomposition under the acid reaction conditions. At room temperature, protamine and the simple guanidines bind approximately half of the maximal amount of formaldehyde in ten to twenty days.[10] Representative experiments are summarized in Table I. These include only studies on water-soluble protamine-formaldehyde derivatives. The insoluble products obtained at higher protamine concentrations contained as much as 0.46 mole of formaldehyde per mole of nitrogen (22% of the weight of the derivative).

The addition of formaldehyde to the guanidyl groups of protamine decreased the basicity of these groups. This was demonstrated by a recently developed method based on the capacity of proteins and polypeptides to bind an acid dye, Orange G, at *p*H 2.2.[11] The basic groups of protamine sulfate were depressed from 41–44 g. equivalents per 10^4 g. to about 30 g. equivalents in products reacted with 4% formaldehyde in acetic acid, and to 13–19 when the reaction had been performed at neutrality. The fact that the basic groups were not completely abolished suggests the interpretation that only those guanidyl groups that bound two methylol groups lost their basicity to such an extent as to be no longer able to bind the dye.[12]

The guanidyl nitrogen in proteins has been shown to be split off almost quantitatively, together with the primary amino and amide nitrogen, when proteins are treated for twenty-four hours with nitrous acid in the presence of 2 *N* hydrochloric acid.[13] In agreement with expectation, protamine yielded 61% of its total nitrogen under these conditions. However, protamine treated with formaldehyde at neutrality yielded only 10–34% of its nitrogen. Similar differences were obtained between the simple guanidines and their formaldehyde derivatives. The latter yielded 20–55% of their total nitrogen, in contrast to 75–90% for the untreated substances (Table I).

All the facts mentioned above can be interpreted as due to the transformation of the guanidyl groups of protamine to guanido mono- and dimethylol groups. However, the observation that the reaction product obtained at *p*H 6–7 differed from untreated protamine in being no longer completely dialyzable suggests that some of the methylol groups have undergone intermolecular condensations to yield material of greater molecular weight. Preliminary osmotic pressure measurements, performed by D. K. Mecham of this Laboratory by Bull's method,[14] indicated average molecular weights of 10,000 to 11,000 for the non-dialyzing fraction of formaldehyde-treated protamine. Untreated protamine is believed to have a molecular weight of approximately 4000.[15] These studies are being extended to include other proteins. The analyses performed on the methylguanidine–formaldehyde reaction product also suggest the occurrence of intermolecular condensation. The properties of the arginine-formaldehyde product described by Smith, *et al.*,[6b] conform with such an interpretation.

It appeared important to exclude the possibility that secondary valences, rather than covalent methylene bridges, might be responsible for the observed increase in the molecular weight of protamine. To this end the protamine-formaldehyde product was exposed to a variety of conditions or reagents known to favor dissociation of secondary valences. As Table II shows, evidence for dissociation was obtained only upon treatment with strong acid. However, the observed increase in dialyzability after exposure to acid was apparently due to hydrolysis of covalent links, since

TABLE II

STABILITY OF A PROTAMINE-FORMALDEHYDE DERIVATIVE

Treatment of derivative[a]	Non-dialyzable nitrogen,[b] %	Fraction of total formaldehyde free in solution, %
None	43, 39	16
2 *N* hydrochloric acid, 23°, 24 hr.	31, 19	49
2 *N* sodium hydroxide, 23°, 24 hr.	53, 47	17
6 *M* guanidine hydrochloride, 23°, 24 hr.	39	6
3 *M* sodium chloride, 23°, 24 hr.	44	16
No additions, 70°, 24 hr.	40	25
No additions, 23°, 1 month	30	18
Dialysis, 23°, 5 days (running water, 4 days)	31	

[a] A detergent, Nacconal NRSF, was also used. It rendered both treated and untreated protamine non-dialyzable, and precipitated upon addition of the acetate buffer used in determining free formaldehyde in solution. [b] Six hours against running tap water; forty hours against distilled water.

(10) Wadsworth and Pangborn (*J. Biol. Chem.*, **116**, 423 (1936)) found guanidine to react slowly and incompletely, when treated with one equivalent of formaldehyde at *p*H 7.8–8.4 and 39°.

(11) Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, **154**, 239 (1944).

(12) No appreciable amounts of formaldehyde were released from the protamine derivative during the dye test. This result is in contrast to the behavior of formaldehyde derivatives of other proteins, from which part of the formaldehyde bound to their amino groups is freed under these conditions.

(13) Plimmer, *J. Chem. Soc.*, **127**, 2651 (1925); Fraenkel-Conrat, Cooper and Olcott, THIS JOURNAL, **67**, 314 (1945).

(14) Bull, *J. Biol. Chem.*, **137**, 143 (1941).

(15) There appear to be no recent observations on the molecular weight of salmine. According to Svedberg and Pedersen ("The Ultracentrifuge," Clarendon Press, Oxford, 1940, p. 236) and Waldschmidt-Leitz, Ziegler, Schaeffner and Weil (*Z. physiol. Chem.*, **197**, 219 (1931)) the molecular weight of clupein is either about 2000 or 4000. The minimal molecular weight of salmine is 2855, according to Waldschmidt-Leitz, *et al.*

part of the bound aldehyde was released during this treatment. Alkali, on the other hand, seemed to favor condensation. It thus appears that the molecular weight of protamine is increased through methylene bridges and that the process can be reversed only concurrently with the hydrolytic liberation of much of the formaldehyde.

Aldehyde analyses performed in conjunction with this experiment indicated the degree of stability of the methylol guanidine bond (Table II). The partial resistance to hydrolysis is borne out by the behavior of these compounds under the conditions of the Plimmer technique[13] and of the dye test.[11] Also, the various methods used for removal of the excess formaldehyde caused little loss of formaldehyde from the derivatives of protamine and other substituted guanidines. Hence, in contrast to other proteins[3] and free unsubstituted guanidine, these compounds contained quite similar amounts of bound formaldehyde under equilibrium conditions and after isolation (Table I).

Guanido-methylol compounds also differ from both amino and amido-methylol compounds in their relative stability in dimedon solution at *p*H 4.6 at room temperature. While the amino-methylol bond is decomposed at a rate which generally exceeds that of the condensation of the liberated aldehyde with dimedon, and while the amido-methylol bond is completely stable under these conditions,[3] a slow liberation of up to 15% of the bound formaldehyde could be demonstrated in the derivatives of protamine and of the simple guanidines when these were treated with dimedon for various time periods ranging from four hours to four days. Since four hours suffice for the quantitative precipitation of formaldehyde from the standard formaldehyde solution, reaction mixtures in which small amounts[16] of free aldehyde were to be determined in the presence of bound aldehyde were therefore allowed to stand for only four hours with dimedon before filtration. It must be assumed that at least part of the "free" aldehyde which was found to contaminate the solutions of the reaction products to an extent of 2–15% of the amount bound, regardless of the number of washing cycles, was actually released during the test. This was unavoidable in the case of arginine, the alpha-amino methylol bond of which may be presumed to resemble that of glycine and alanine in being split quantitatively in the presence of dimedon.[3,10] Thus the amounts of bound formaldehyde may actually have been about 5–10% higher than those computed by subtraction of the "free" from the total aldehyde (Table I).

(16) Complete precipitation of free formaldehyde was not achieved in four hours in reaction mixtures containing much free aldehyde in the presence of little that was bound. These were filtered therefore after twenty to twenty-four hours of standing.

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Summary

Guanidyl groups bind formaldehyde at 70° in neutral or acid solution. Protamine sulfate, as well as simple substituted guanidine salts, bind up to 2 moles for each guanidine group; free guanidine salts up to 3 moles of formaldehyde. At room temperature the reaction proceeds slowly and to a lesser extent.

The reactivity of the guanidyl nitrogen toward nitrous acid in mineral acid is decreased after combination with formaldehyde. A decrease in the basicity of the guanidyl groups is also evident.

If the reaction is performed at protamine concentrations exceeding 3%, the product is largely water insoluble. In 1–3% solution and at *p*H 6–7, the reaction product remains soluble, but differs from untreated protamine in being in part non-dialyzable. Osmotic pressure measurements indicate a considerable increase in the average molecular weight of these treated preparations. This is regarded as evidence for intermolecular condensation through crosslinking methylene groups.

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