[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. XXII. A Crystallizable Guanidinated Derivative of Human Serum Albumin[1a,b]

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While various physical chemical techniques have made possible the approximate description of the shapes of protein molecules as geometrical solids, the polypeptide arrangement responsible not only for the geometry but also for the chemical properties of proteins remains obscure. One approach which may shed some light on this "fine structure" of the protein molecule lies in the preparation of series of derivatives of protein molecules. In order to simplify interpretation of the results it is desirable that such derivatives should be as homogeneous as the starting material. In practice this means that reagents must be chosen each of which is specific for one or more of the various reactive groups of the protein, and conditions must be found where the reaction can proceed quantitatively and without the introduction of non-specific structural changes or denaturation.

The following studies on the reaction of O-methyl isourea with human serum albumin were undertaken to determine to what extent this reaction might fulfill the above requirements. Previous investigations by Greenstein and others[2,3,4] on the reaction of O-methyl isourea with lysine and lysylglutamic acid indicated that this reagent reacted readily with the ϵ-amino group of peptides. Preliminary studies on the effect of this reagent on horse and bovine serum albumins indicated that a marked decrease in solubility accompanied the conversion of the amino groups to guanidino groups.[5,6]

(1) (a) This paper is Number 78 in the series "Studies on the Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross. This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August, 1941, to July, 1946, it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institute of Health. (b) Presented, in part, before the Division of Biological Chemistry of the American Chemical Society at New York City in September, 1947.

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(2) J. P. Greenstein, *J. Biol. Chem.*, **109**, 529, 541 (1935).

(3) J. P. Greenstein, *J. Org. Chem.*, **2**, 480 (1938).

(4) See also J. Kapfhammer and H. Müller, *Z. physiol. Chem.*, **225**, 1 (1934).

(5) E. J. Cohn, *Chem. Revs.*, **28**, 395, footnote 10 (1941).

(6) Schütte has similarly used S-methyl isothiourea: *Z. physiol. Chem.*, **279**, 52, 59 (1943).

Materials and Methods

Human serum albumins used in these studies were crystallized by the methods already described[7] and contained less than 0.1% of globulins by immunological test. Three preparations were used. Two of them (179-4X and 179-5X) were derived from the same preparation of normal human serum albumin after it had been crystallized four and five times, respectively, with the aid of chloroform. Another preparation of normal human serum albumin (Dec. 10) had been crystallized three times with the aid of *n*-decanol.

O-Methy isourea hydrochloride,[8] recrystallized from hot ethanol and thoroughly dried, was used in most of the studies here reported.

O-Methyl isourea acid sulfate replaced the hydrochloride in our most recent studies, since it proved more stable, more readily purified, and more suitable for the preparation of the free base. The preparation and properties of this compound appear not to have been reported previously and are therefore described here:

To an aqueous solution of crude methyl isourea hydrochloride was added an excess of sodium picrate solution. After standing overnight at 0° the methyl.isourea picrate was filtered, washed with cold water and recrystallized from solution in the minimum quantity of boiling water by cooling to 0°. The crystals, washed and dried at 110°, had a m. p. of 189° (dec.); recorded, 184° (dec.).[9]

For preparation of the acid sulfate, the methyl isourea picrate was dissolved in the minimum quantity of boiling acetone; the solution was then chilled and concentrated sulfuric acid, slightly in excess of theoretical (0.25 cc./g. of picrate), added with stirring. The crystalline acid sulfate was filtered, washed with acetone and then with ether. It was recrystallized by dissolving in 5 parts of absolute methyl alcohol and precipitating with a mixture of 10 parts of acetone and 10 parts of ether. The crystals, washed with ether and dried at 50–60° for twenty minutes, had a m. p. of 119–120°. The neutralization equivalent (methyl red as indicator) was 174 (calcd. for $C_2H_8O_5N_2S$ = 172); nitrogen determination by Kjeldahl, 15.9 (calcd. for $C_2H_8O_5N_2S$, 16.3).

Preparation of Guanidinated Serum Albumin.—In general the chemical modification of a protein can be carried out most safely at the lowest possible temperature, since the temperature coefficient of denaturation is much larger than that of most chemical reactions. In these experiments guanidination was carried out at 0°. For economy, concentrated solutions were employed. As discussed below, the rate of the reaction depended largely on the *p*H. In fact, it

(7) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(8) We are indebted to Dr. R. Bowling Barnes of the American Cyanamid Co. for this preparation.

(9) E. A. Werner, *J. Chem. Soc.*, **105**, 928 (1914).

appeared possible to control the degree of guanidination through the *p*H (see Fig. 3), using the reagent itself as a buffer. The methyl isourea reagent was prepared by the addition of alkali to its salt at 0° immediately before use. At the end of the reaction, the protein was separated from excess reagent and from its decomposition products by dialysis or by isoelectric precipitation, and dried from the frozen state. Unreacted reagent could be recovered as its picrate. Details for the preparation of a crystalline derivative (see Fig. 1) of human serum albumin follow.

Preparation of Crystallized Guanidinated Serum Albumin.—To 17.2 g. (0.10 mole) of methyl isourea acid sulfate dissolved in 50 cc. of water at 0° is added 0.19 equivalent of hot concentrated barium hydroxide, 10 cc. at a time with cooling between successive additions. (5 *N* barium hydroxide, stored in a boiling water-bath, can readily be handled in hot pipets provided the solution is forcibly expelled.) The barium sulfate is centrifuged off, washed repeatedly with water until the total volume of supernatant and washings is 200 cc. (this gives a 0.5 *M* solution). The *p*H of this solution should be between 10.5 and 11 (1:5 dilution).

Forty grams of decanol-crystallized human serum albumin[7] at 0° is dissolved in the methyl isourea solution, and the resulting solution allowed to stand three days at 0°. It is then diluted to 1 liter with water and sufficient 0.02 Γ/2, *p*H 4.0 acetate buffer is added to lower the *p*H to the point of incipient precipitation (approximately *p*H 5.2) and seeded. After allowing three days for crystallization, the crystals are centrifuged, washed with 0.01 *M* sodium chloride, and dried from the frozen state. If the solution fails to crystallize or if the mother liquor contains more than 0.5% protein, more *p*H 4.0 buffer is added in small amounts over the course of a day or longer until maximum precipitation has occurred. This precipitate may then be redissolved in sodium bicarbonate and adjusted with acetate buffer to a protein concentration of 0.5% at *p*H 5.0 and Γ/2 0.02 for crystallization.

The mother liquor, which should contain less than 10% of the total protein, can be treated with an equal volume of 0.2 *M* lithium picrate to recover most of the unreacted methyl isourea, which can be purified as described above.

The following analytical methods were employed to follow the course of the reaction and to test the nature of the product.

Van Slyke amino-nitrogen analyses were carried out at room temperature (24–27°), on dialyzed samples containing approximately 50 mg. of protein, in the manometric apparatus[10] using a thirty-minute reaction time. Protein concentration was determined by drying to constant weight at 105°. Control analyses on normal serum albumin indicated the presence of 64 to 68 amino groups per mole (assuming a molecular weight for serum albumin of 69,000).[11]

Non-protein nitrogen analyses for unreacted reagent were carried out on aliquots of the solution following precipitation of the protein with 10% trichloroacetic acid. The aliquots were digested with sulfuric acid–hydrogen peroxide, nesslerized and measured in a photoelectric colorimeter.[12]

Methanol was determined on samples of distillate by a potassium dichromate oxidation method.[13] The sample was acidified before distillation to stop the reaction.

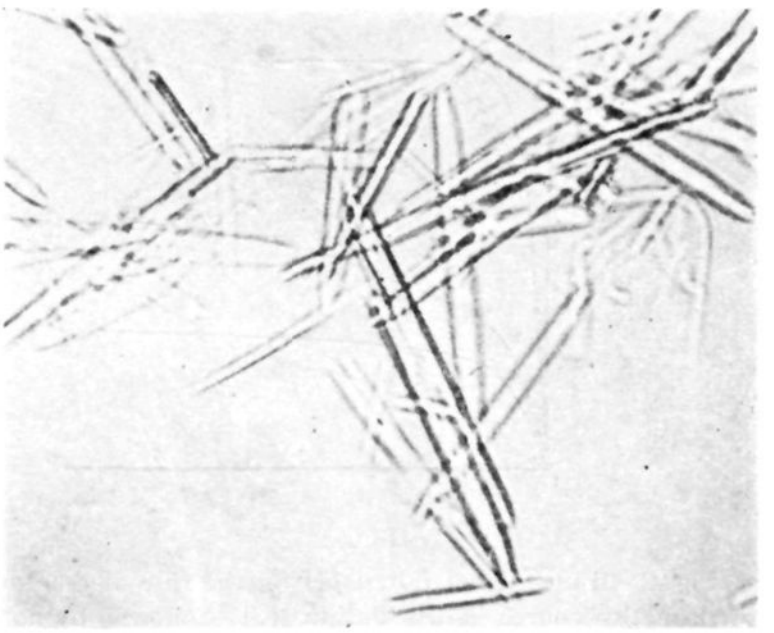

Fig. 1.—Crystals of human serum albumin (with 54 amino groups guanidinated) from 0.02 Γ/2, *p*H 5.0, acetate buffer.

Unreacted methyl isourea was determined as the picrate by precipitation from neutral solution by the addition of an equal volume of 0.2 *M* lithium picrate. After twenty-four hours standing at 0° to complete crystallization, the precipitate was filtered, washed with a small volume of water, dried at 110° and weighed. The application of this method to a series of standard methyl isourea solutions varying in concentration from 0.1 to 0.025 *M* gave recoveries of 88 to 93%.

***p*H measurements** were made at room temperature with a glass electrode (Cambridge research model). Protein samples were usually diluted to 1% with water.

Electrophoretic, ultracentrifugal and viscosimetric analyses, to determine whether the modified protein had been rendered heterogeneous with respect to size, shape or net charge, were carried out by methods already described.[14a,b,c]

Solubility measurements were carried out by adding a neutral concentrated solution of the protein to a volume of acetate buffer calculated to give the desired ionic strength and 1% protein in the system. After standing twenty-four hours at room temperature, the precipitate was separated by centrifugation and the *p*H and protein concentration of the solution determined, the latter by the biuret reaction.[15]

Results and Discussion

Most reactions for the preparation of protein derivatives involve a radical change in the nature of a chemical group—often accompanied by the loss of a charged or ionized group. O-Methyl isourea, however, reacts to replace the basic amino group by the more basic guanidino group. Being more reactive than S-methyl isothiourea and cyanamide,[16] it would appear preferable to these despite the instability of the reagent in aqueous systems. This instability results in the

(10) J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry," Vol. II, Williams and Wilkins Co., Baltimore, Md., 1932, p. 385.

(11) Brand has reported 69 amino groups per mole assuming a molecular weight of 70,000. This figure reduces to 68 for a molecular weight of 69,000 (E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946)).

(12) F. C. Koch, "Practical Methods in Biochemistry," Williams and Wilkins Co., Baltimore, Md., 1941, p. 119.

(13) R. N. Harger, *J. Lab. and Clin. Med.*, **20**, 746 (1934).

(14) (a) S. H. Armstrong, Jr., M. J. E. Budka and K. C. Morrison, THIS JOURNAL, **69**, 416 (1947); (b) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. and Coll. Chem.*, **51**, 184 (1947); (c) These analyses were carried out under the direction of J. L. Oncley by M. J. E. Budka, C. Gordon and E. Ellenbogen.

(15) J. W. Mehl, *J. Biol. Chem.*, **157**, 173 (1945).

(16) Schütte[6] reports that guanidination with S-methyl isothiourea required weeks at room temperature and that cyanamide itself required months.

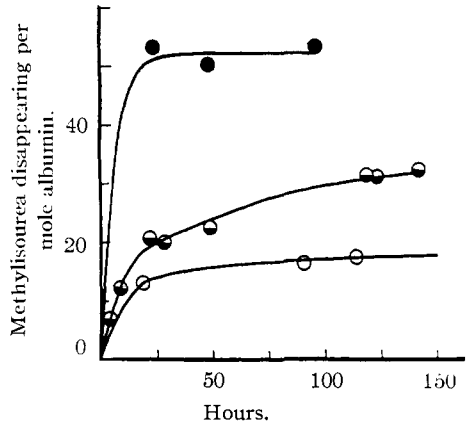

Fig. 2.—Influence of initial *p*H on the rate of reaction with methylisourea hydrochloride at 0° (followed by non-protein nitrogen determinations): ●, *p*H 10.4, 0.50 *M* methylisourea, 17% protein; ◒, *p*H 9.8, 0.22 *M* methylisourea, 18% protein; ○, *p*H 9.2, 0.23 *M* methylisourea, 20% protein.

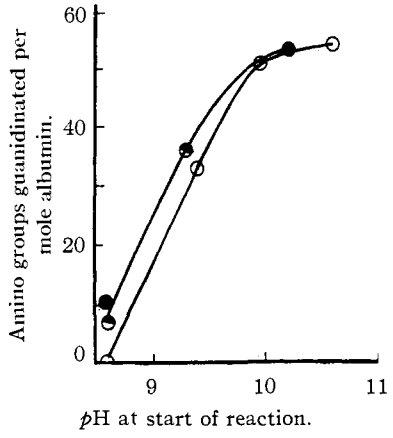

Fig. 3.—Effect of *p*H on the degree of guanidination (disappearance of protein amino nitrogen—Van Slyke) albumin concentration, 15 to 20%: ○, after five days with 0.4 *M*, methylisourea; ●, after five days with 0.8 *M* methylisourea; ◒, after twelve to fourteen days with 1.0 *M* methylisourea.

formation of methyl alcohol and two unidentified crystalline deposits.

The rate of the reaction of methyl isourea with albumin increases rapidly with *p*H (Figs. 2 and 3) indicating that reaction involves the un-ionized form of one or probably both of the reactants. Thus for quantitative reactions it would appear desirable to operate at the most alkaline reaction, up to *p*H 11, at which the protein is stable. Since serum albumins rapidly gel in the neighborhood of *p*H 11 even at 0°, we have limited our studies to the *p*H range 8.5–10.5. We have no evidence of hydrolytic changes in this range. Thus, serum albumins do not liberate ammonia at 0° even at *p*H 11.

When partial guanidination is desired, it would appear possible to control the reaction most readily through the *p*H (Figs. 2 and 3). However, some variation in ease of reaction may occur from preparation to preparation. Thus, with a less stable preparation (179-5X) 37 groups reacted at *p*H 8.8 and 48 groups reacted at *p*H 9.3. Whether the *p*H controls the extent of guanidination only by controlling the reaction rate, or whether the availability of the amino groups also plays a role, cannot be decided at this time. However, the apparent plateaus shown in Fig. 2 are at least partially the result of the shift in *p*H which accompanies the reaction (Table I). In other experiments carried out below *p*H 10 even larger shifts of as much as 0.5 *p*H unit have been observed.

TABLE I

CHANGE IN *p*H OF THE REACTING MEDIUM AS A FUNCTION OF THE INITIAL *p*H

Albumin concn.	Methyl iso-urea, *M*	KOH, *M*	*p*H[a] Initial	After 2 days at 0°	Δ*p*H
0	0.1	0.05	9.88	9.90	+ .02
	.1	.08	10.40	10.39	− .01
0.002 *M* (14 g./100 cc.)	0	0.17	11.35	11.34	−0.01
0.002	.1	.15	10.06	9.90	− .16
	.1	.17	10.26	10.19	− .07
	.1	.18	10.43	10.36	− .07
	.1	.20	10.61	10.60	− .01
	.1	.24	11.14	11.20	+ .06

[a] *p*H measured in the glass electrode at 24° on a 1:10 dilution. In order to increase the precision of *p*H measurement, 1-cc. aliquots of the samples were removed immediately after preparation and stored at −40 to −50° during the two-day reaction period, to inhibit the reaction. They were then diluted and the *p*H measured at the same time as the reacted samples, the "initial" and the "after 2 days" samples being read successively in the *p*H meter. Measured in this way, a change of greater than 0.02 *p*H unit is significant.

This shift in *p*H is predicted by the equations

$$\begin{array}{ccccc} CH_3O{-}C(NH_2)_2^+ & + & Prot\text{-}NH_3^+ & \longrightarrow & Prot\text{-}NH{-}C(NH_2)_2^+ + CH_3OH + H^+ \\ \uparrow\downarrow & & \uparrow\downarrow & & \\ CH_3O{-}C(NH)NH_2 & + & Prot\text{-}NH_2 & \longrightarrow & Prot\text{-}NH{-}C(NH_2)_2^+ + CH_3OH - H^+ \end{array}$$

The equation is written in this form because in the neighborhood of *p*H 10, where reaction takes place readily, appreciable quantities of the reactants must exist in both ionized and un-ionized states, whereas the much more basic reaction product (the guanidino group) must exist almost exclusively in the ionized state. It will be seen that the reaction frees protons at a *p*H below the mean *pK* of the reactants and absorbs protons at a *p*H above this (*i. e.*, since the *pK* of O-methyl isourea hydrochloride is 9.7, and the *pK* mean of the ammonium residues of serum albumin is approximately 11.1,[17] the *p*H should remain con-

(17) Estimated with the hydrogen electrode from the titration curve of serum albumin under similar conditions by Charles Tanford. Personal communication.

stant when the reaction is carried out at *p*H 10.4). The data in Table I do, in fact, show the predicted behavior—no *p*H change being observed at *p*H 10.6.

Balance sheet studies comparing the amount of reagent disappearing from solution with the number of amino groups disappearing from the protein, show good correlation (Table II), the discrepancies lying well within the experimental error. Thus, of the groups in serum albumin available for reaction (Table III), the ϵ- and α-amino groups would appear to be the only ones involved.

TABLE II

STOICHIOMETRY IN THE REACTION OF METHYL ISOUREA WITH HUMAN SERUM ALBUMIN

	Initial Concn. (M)			Final Concn. (M)		Reactants/ mole protein	
Prepn.	Albumin	*p*H	Me isourea (HCl)	Me isourea (NPN)	NH_2/ Mole prot. (HNO_2)	NH_2	Me isourea
F	0.0025	9.2	0.23	0.18	49	15	20
B	.0026	9.8	.215	.129	30	34	33
H	.0029	10.2	.51	.37	7	57	49

TABLE III

REACTIVE GROUPS IN HUMAN SERUM ALBUMIN[a] (MOL. WT. = 69,000)

Group	Number[b]	Group	Number[b]
Epsilon amino	58	Sulfhydryl	(1)[c]
Terminal amino	9	Disulfide	18[d]
Guanidino	24	Methionyl	6
Tyrosyl	18	Hydroxyl	53
Imidazole	16	Amido	43
Carboxyl	93	Indole	1

[a] Hydrocarbon residues and the peptide links have been excluded. [b] Calculated from the analyses of Brand and co-workers,[11] with the exception of the estimate for the sulfhydryl group. [c] Unpublished observations by one of us (W. L. Hughes, Jr.) based on reactivity of serum albumins with mercury compounds. Brand finds 4 cysteines per mole of hydrolyzed protein. [d] On the basis of footnote [c] the cysteine sulfur reported by Brand has been included with the cystine sulfur.

Whether only ϵ-amino groups react with methyl isourea cannot be definitely stated. Exhaustive reaction with methyl isourea has led to the coverage of 54 to 57 amino groups (estimated by Van Slyke analysis). Since not more than 9 of these could have been α-amino groups, it would seem evident that at least a majority of the 58 lysyl residues must have reacted.[18] It is suggestive that the small amount of amino nitrogen remaining following the most vigorous treatment employed is approximately equivalent to the α-amino groups.[19]

The following properties of guanidinated serum albumins have been investigated.

(18) Bioassay for lysine in hydrolysates of our products has shown a decrease in lysine content at least as great as the decrease in amino nitrogen (E. Brand, personal communication).

(19) By means of the Sakaguchi reaction, Greenstein showed that O-methyl isourea reacted preferentially with the ϵ-amino group of lysylglutamic acid.[2] However, α-guanidino acids have been prepared by treating α-amino acids with O-methyl isourea.[4,6]

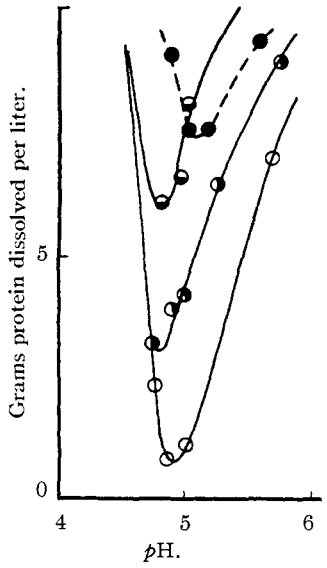

Fig. 4.—Solubility of guanidinated serum albumins in acetate buffers at 25°: total protein concentration 10 g./liter; dashed line, albumin with 32 amino groups guanidinated; solid line, 51 amino groups guanidinated; ◒, 0.1 Γ/2; ◑, 0.05 Γ/2; ○, ●, 0.01 Γ/2.

The solubility of serum albumin decreased sharply on guanidination so that the product behaved like a euglobulin[5] (Fig. 4). The solubility was smaller the larger the number of amino groups converted to guanidino groups. Figure 4 shows relative solubilities after twenty-four hours equilibration. If the saturated solutions, from which the precipitates had been separated, were allowed to stand for several days, a portion of the protein remaining in solution frequently separated in crystalline form.

The relative viscosity of a 4% solution of guanidinated (54 groups) serum albumin in *p*H 7.0, Γ/2 0.15 phosphate buffer was 1.21 as compared with 1.20 for the starting material.

In the Tiselius apparatus, guanidinated preparations, like the starting material, appeared completely homogeneous at *p*H 8.6 in 0.1 Γ/2 barbiturate buffer. The mobility at this *p*H appeared slightly greater than that of normal serum albumin although the increase was hardly greater than the experimental error.

In the ultracentrifuge, about 5% of material sedimenting faster (*s ca.* 6.5) than normal serum albumin was observed both in the original unmodified albumin and in the guanidinated material. No significant change was observed in the amount of this faster sedimenting material, or in the rate of boundary spreading (apparent diffusion constant) for any of these preparations. The sedimentation constant also appeared unchanged. (The increase in molecular weight from 69,000 to 71,000 upon the addition of 50 $C(NH_2)_2$ groups would be hardly detectable by this means.)

The extinction $E^{1\%}_{1cm.}$ at 280 mμ was 5.0 for

human serum albumin with 54 amino groups guanidinated. (Assuming this modification has no effect on the extinction except that due to the increase in molecular weight mentioned above, the extinction of normal human serum albumin should have been decreased from 5.3 to 5.1 following guanidination.) The optical rotation $[\alpha]^{25}_{5461}$ of the same guanidinated product was $-65°$, a significant decrease from the value of $-78°$ found for normal human serum albumin.

The homogeneity of guanidinated serum albumin may be considered both as evidenced by the usual physical chemical criteria of homogeneity and as implied by the stoichiometry of the reaction. Assuming that one started with a pure protein (one component) and reacted all the groups of one type in the protein in the same manner, the final product should be homogeneous, although the intermediates might be very heterogeneous.[20]

Since it has not proven possible to convert the amino groups of serum albumin quantitatively to guanidino groups, heterogeneity in the fine structure of the products cannot be excluded. However, the few remaining amino groups in the most guanidinated products would appear to be of a very different reactivity from the rest, and therefore the heterogeneity must be of a much lower order than that predicted on a purely statistical basis.

Unfortunately serum albumin itself does not fulfill the phase-rule requirements of a pure substance (*i. e.*, constant solubility with variation of the amount of saturating body). Consequently, guanidinated albumin could not be expected to meet this most rigorous test. However, when analyzed in the ultracentrifuge, in the electrophoresis apparatus, and in the viscosimeter, guanidinated albumins appear no less homogeneous than the starting material, a conclusion which is markedly strengthened by the crystallizability of highly guanidinated products.

(20) Herriott and Northrop would appear to have prepared a homogeneous acetyl derivative of pepsin. They found that with limited amounts of ketene the four amino groups could be quantitatively and specifically covered: *J. Gen. Physiol.*, **18**, 35 (1934).

Summary

1. Human serum albumin reacts with O-methyl isourea with the formation of guanidinated derivatives.

2. The preparation and properties of methyl isourea acid sulfate are described.

3. The reaction appears specific for the amino groups of serum albumin, largely converting the lysine residues into homo-arginine residues.

4. By controlling the *p*H, the proportion of the 68 amino groups converted to guanidino groups can be varied from less than 10 below *p*H 9 to more than 50 above *p*H 10.

5. The preparation and properties of a crystallized guanidinated derivative of human serum albumin are described.

BOSTON 15, MASS. RECEIVED DECEMBER 22, 1948

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF ILLINOIS]

The Determination of the Decomposition Pressures of Certain 1,10-Phenanthroline Hydrates

BY JAMES S. FRITZ,[1] F. WM. CAGLE, JR.,[2] AND G. FREDERICK SMITH

The recognition of the hydrogen bond and appreciation of its extent were due to Latimer and Rodebush,[3] who used this concept to explain abnormally high dielectric constants for water and hydrogen fluoride, the small ionization of ammonium hydroxide and the dimerization of certain carboxylic acids. Since then the hydrogen bond (or hydrogen bridge) concept has been used to explain qualitatively many abnormal properties of organic compounds.[4] The instances in which the strength of a hydrogen bond has been estimated quantitatively are, however, not so numerous. From the heats of dimerization, the O—H...O bond strength in formic acid has been estimated to be 7.2 cal./mole[5] and in acetic acid to be 7.25 cal./mole.[6] For similar bonds in *o*-hydroxybenzoic acid and benzoic acid, values of 4.7 and 4.3 cal./mole, respectively, have been found.[7] The strength of the hydrogen bonds in methanol and ethanol has been estimated to be about 6.2 cal./mole.

1,10-Phenanthroline as well as the 5-bromo and 5-methyl derivatives form monohydrates. Infrared data indicate that the water molecule is held through two hydrogen bonds to the ring nitrogens.[8] These hydrates are definite chemical entities with sharp and reproducible melting points. When 1,10-phenanthroline monohydrate is heated, it dissociates reversibly and rapidly into 1,10-phenanthroline and water.

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(3) Latimer and Rodebush, THIS JOURNAL, **42**, 1419 (1920).

(4) Huggins, *J. Org. Chem.*, **1**, 407 (1936).

(5) Halford, *J. Chem. Phys.*, **10**, 582 (1942).

(6) Ritter and Simons, THIS JOURNAL, **67**, 757 (1945); Halford, *J. Chem. Phys.*, **9**, 859 (1941); **10**, 582 (1942).

(7) Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, N. Y., 1944, pp. 288-289.

(8) Smith and Richter, "Phenanthroline and Substituted Phenanthroline Indicators," G. Frederick Smith Chemical Company, Columbus, Ohio, 1944.