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A STUDY OF THE SULFHYDRYL GROUP IN BOVINE ALBUMIN*

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SUMMARY

1. The binding of ^{110}Ag by bovine albumin has been studied by the technique of equilibrium dialysis at pH 3, 6 and 10.

2. The reactivity of the sulfhydryl group of bovine albumin with silver ion at pH 6 has been found to be smaller than expected by a factor of at least 10^4.

3. The action of ammonia at pH 10 upon the bovine albumin sulfhydryl group seems to be specific in greatly increasing its reactivity with silver ion.

4. Isolation of the reaction product, CM-cysteine, from bovine albumin, treated with iodoacetamide and subsequently hydrolyzed, demonstrated that bovine albumin does contain the sulfhydryl group in some form.

5. These observations form the basis for an hypothesis that the sulfhydryl group in bovine albumin exists in the form of an isothiuronium-type compound in the native state of the protein.

INTRODUCTION

In the past interest in the sulfhydryl group of proteins and enzymes has been primarily limited to means for its quantitative measurement; little attention has been given to the actual chemistry of this group in its native form in enzymes and proteins.

The present study was the outgrowth of an attempt to develop a better and more reliable method for the measurement of the sulfhydryl group in its native form in proteins and enzymes. The equilibrium dialysis technique was chosen as a relatively mild procedure for the direct study of the interaction of protein sulfhydryl with very low concentrations of $^{110}Ag^+$. The method did not prove of value in the measurement of the sulfhydryl group, *per se*, in the one protein, BA, but did yield results permitting a reevaluation of the concept of the nature of the "masked" sulfhydryl in BA. An hypothesis has been formulated upon the basis that the sulfhydryl group exists bound to some other group in the molecule. That this group is the guanidine residue of

Abbreviations: BA, bovine albumin; IA, iodoacetamide; CMC, carboxymethyl-cysteine.

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arginine to form an isothiuronium-type group is strongly suggested from analogies in the known chemistry of isothiuronium and thiazoline compounds.

EXPERIMENTAL

Materials

Crystalline BA was obtained mainly from Armour and Company (Chicago, Ill.); a few experiments were carried out with BA obtained from Pentex Laboratories (Kankakee, Ill.). A molecular weight of 69000 was assumed for this material.

IA-treated BA was prepared in the following manner: To a solution of BA in 0.01 M phosphate (pH 8) was added the desired amount of IA (usually two moles per mole of protein). After standing for 15–18 h this solution was dialyzed once against 100 vol. of 0.001 M EDTA, disodium salt, followed by replacement of the external solution with ion-free water two more times. The resulting protein solution was either used immediately in experiments or was lyophilized and stored in the cold until used.

IA labelled with ^{14}C was prepared in the following manner: To 2 mmoles of methylbromoacetate, labelled in the carboxyl carbon (obtained from Isotope Specialties Co., Glendale, Calif.) with a specific activity of 0.01 mC/mmole, was added 2 ml of conc. NH_4OH over a period of 30 min while being kept at $-10°$. The white precipitate of bromoacetamide which separated, after recrystallization from benzene, was converted to IA by treatment with sodium iodide in acetone at room temperature. IA was obtained by removal of the sodium bromide and drying of the acetone solution. Three recrystallizations from chloroform afforded a compound of constant specific radioactivity during the last two recrystallizations whose melting point was 90.5–91.5°.

Ion-free water was used routinely in the experiments described; it was prepared by passing distilled water through an Amberlite XE-81 (MB-1) column.

The glassware used was washed and rinsed in distilled water, soaked in 0.001 M EDTA (tetrasodium salt) and thoroughly rinsed with ion-free water.

METHODS

Amperometric titration

Amperometric titrations were carried out with the rotating platinum electrode in 0.3 M NH_3–NH_4NO_3 buffer (pH 10) according to the method of KOLTHOFF AND HARRIS[1].

Isotope counting

The radioisotope of silver used, ^{110}Ag, is a strong β- and γ-ray emitter and has a half-life of approx. 250 days. It was obtained from Oak Ridge National Laboratory (Oak Ridge, Tenn.) as 19.4 ml of 0.55 M $AgNO_3$ containing 1.55 mC of activity per ml. For use in experiments, this stock solution was diluted with ion-free water to approx. 0.05 M solution.

Counting of solution aliquots containing ^{110}Ag was done either in a deep-well Geiger–Mueller γ-ray counter or in a Nancy Wood head, well-type scintillation counter. All pipettes used to transfer aliquots for counting were counted with the tube containing the aliquot, since it was found that a considerable percentage of the silver ion in dilute solutions remains bound to the glass pipette*. 0.5-ml blowout

* As much as a third of the silver ion present in a 10^{-5} M solution was bound to the surface of the glass pipettes.

pipettes were the type most often used. Of this type those were selected which contained the prescribed volume in a minimum pipette length in order to minimize error from silver clinging to the glass outside the volume counted by the counter.

Aliquots of solutions with dissolved compounds containing ^{14}C were dried in aluminum cups and counted in a windowless gas-flow counter. Self-absorption of emitted radiation under the conditions of these experiments was negligible.

Because of the volatility of free IA, it was found necessary to allow solutions containing this substance to react with cysteine at pH 10 (NH_4OH) before drying for counting.

Decontamination of glassware

Glassware exposed to radioactive silver was decontaminated by soaking in a dilute $HF-HNO_3$–detergent mixture according to the procedure of CRAWLEY[2]. Another method involving equilibrium of glassware with non-radioactive silver solution followed by rinsing in NH_4OH proved far less effective.

Equilibrium dialysis experiments

Treatment of dialysis tubing: cellophane dialysis tubing contains sulfur compounds incorporated in the processing of the cellulose, which bind silver ion very strongly, an observation also reported by BENESCH, LARDY AND BENESCH[3] and by HUGHES AND KLOTZ[4]. Removal of 90 % of this binding capacity was accomplished by treatment with methyl iodide in the following manner: Glass beads were placed in a round-bottom flask containing 1 mmole methyl iodide per 2 cm^2 of dialysis tubing. The flask was filled two-thirds full with a solution containing an amount of Na_2HPO_4 equivalent to the methyl iodide; dialysis tubing was then immersed in the solution, the flask fitted with a reflux condenser, and heated at 80° for 48 h. After this treatment the tubing was washed thoroughly with ion-free water and stored under water in the cold.

Equilibrium dialysis: Treated dialysis bags were filled with 2.0 ml of 10^{-5} M BA and suspended by a polyethylene string (cut from heavy sheeting) in a buffered solution containing radioactive silver nitrate sufficient to give the desired concentration of silver ion after equilibration. Solutions were placed in polyethylene bottles. The bottles were enclosed in aluminum foil to exclude light and placed upon a mechanical shaker for 3–4 h. Preliminary experiments indicated that dialysis equilibrium was established within 3 h under these conditions. After equilibration aliquots were removed for counting from the outside solution, and the protein solution inside the dialysis bags. A bag containing only buffer was included in each bottle to obtain a test of whether equilibrium had been reached. All experiments were run in duplicate.

Hydrolysis and ion-exchange chromatography of BA hydrolysate

1 g BA treated with radioactive IA as described above was heated under vigorous reflux for 24 h with 200 ml of 6 N HCl (C. P. reagent grade). After hydrolysis excess HCl was removed under reduced pressure while heating with a steam bath. After treatment residual water and HCl were removed under high vacuum using liquid nitrogen and solid KOH traps.

The dry hydrolysate was dissolved in approx. 4 ml of ion-free water and the pH adjusted to 6.0 with 11 M NaOH, whereupon a precipitate of tyrosine and cystine separated. The solution was cooled to 0°, the precipitate removed by filtration and

the precipitate washed 3 times with 1-ml portions of cold ion-free water. No radioactivity was found in this precipitate. Fractionation of the amino acids in the filtrate was accomplished by ion-exchange chromatography upon 60 ml of Dowex-1 (200–400 mesh, 10% DVB) in the formate form.

In this system monocarboxylic amino acids pass on through the column and dicarboxylic acids are adsorbed. Gradient elution of the dicarboxylic acids was accomplished with 0.5 M formic acid in the upper reservoir and 300 ml of initially ion-free water in the lower one.

Identification of CMC: CMC, the compound expected from the hydrolysis of the reaction product of IA with the cysteine residue of BA, was identified by descending filter paper chromatography using 80% phenol–water. It was compared with CMC synthesized according to the method of MICHAELIS AND SHUBERT[5].

RESULTS

Preliminary experiments studying the binding of silver ion to BA equilibrated with silver halide suspensions, excess halide ion controlling the free silver ion concentration gave non-reproducible results; this approach was abandoned as a means of studying silver ion–BA interaction in favor of the equilibrium dialysis technique similar to that employed by KLOTZ[6] to study cation binding to proteins.

The equilibrium-dialysis technique proved to be a satisfactory and reproducible method for studying BA–silver ion interaction. The results obtained for a number of experiments are given in Fig. 1 in which the amount of silver bound to BA at

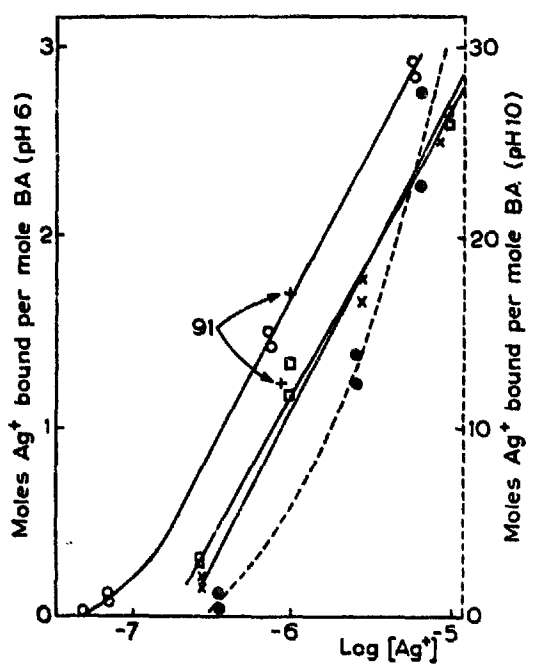


Fig. 1. The binding of silver ion by Armour BA. The ordinate on the left applies to experiments at pH 6 in 0.05 M citrate buffer (solid curves and experiment designated 91). The ordinate on the right applies to an experiment at pH 10 in 0.05 M borate buffer (broken curve).

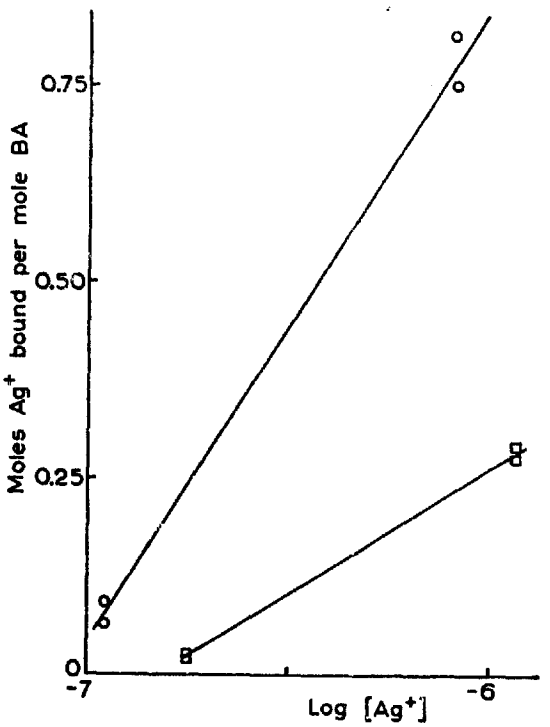


Fig. 2. Effect of 6 M urea on the binding of silver ion to BA in 0.05 M citrate buffer (pH 6); urea placed outside dialysis bags at beginning of equilibration. O—O, buffer alone; □—□, 6 M urea + buffer.

pH 6 and pH 10 is the ordinate and the negative logarithm of the silver ion concentration is the abscissa. The higher of the two points marked 91 in Fig. 1 is the result of an experiment in which 10^{-5} M BA solution containing $3 \cdot 10^{-4}$ M silver ion was placed inside the bag initially and the silver ion allowed to dialyze out—the reverse of the usual equilibrium dialysis technique. The lower point is the same experiment in which silver ion dialyzed out of the first bag and into a second containing a BA solution identical to the first.

Also in Fig. 1 is presented the result of an experiment in which BA was equilibrated with various concentrations of silver ion at pH 10 in 0.05 M borate buffer.

TABLE I

EQUILIBRIUM DIALYSIS OF BA WITH SILVER–AMMONIA BUFFER SYSTEM

A

Buffer system: 0.3 M NH_3–NH_4NO_3 (pH 10)

Titre of BA by amperometric titration	Equilibrium dialysis (moles silver bound/mole BA)
0.76	0.68

B

Expt.	*Treatment*	*Mole Ag^+ bound/mole BA*
1	Equilibration directly with 0.05 M citrate buffer (pH 6) containing finally $3.7 \cdot 10^{-7}$ M Ag^+	0.59
2	Equilibration with 0.3 M NH_3–NH_4NO_3 buffer (pH 10) containing $9.1 \cdot 10^{-6}$ M Ag^+: $[Ag^+]$ free, calculated = $9 \cdot 10^{-12}$ M	0.91
3	Equilibration as in Expt. 2 followed by dialysis against 2 portions of 0.05 M citrate buffer (pH 6) containing finally $3.7 \cdot 10^{-7}$ M Ag^+	1.86
4	Treatment with 2 moles of IA and equilibrated with silver–ammonia system as in Expt. 2	0.12

In Table I, part A, are presented the results of an experiment to determine if BA, when dialyzed to equilibrium against the same buffer used in the amperometric titration procedure, would bind an amount of silver equivalent to the amperometric method titre; part B of Table I describes an experiment to determine if the effect is reversible. One aliquot of a BA solution, when equilibrated with ammonia buffer and silver, did not return to the control value of 0.59 mole bound per mole of protein, but increased to 1.86 moles of bound silver upon exposure to citrate buffer (pH 6) containing the control amount of silver ion. BA treated with 2 moles IA per mole BA bound only 0.12 mole of silver in the silver–ammonia buffer system. In another experiment at pH 6 in citrate buffer containing 0.25 M NH_4NO_3 no significant effect of ammonium ion on the binding of silver ion was noted.

In Fig. 2, are presented the results of an experiment to determine the binding pattern of silver ion to BA at pH 6 in the presence and absence of a denaturing agent, 6 M urea. BA bound considerably less silver ion in the presence of high concentrations of urea than in its absence. In this experiment the binding to protein in the absence of urea is less than normal, apparently due to the somewhat lower sulfhydryl content of this BA lot as determined by amperometric titration.

When BA was treated with 6 moles of IA per mole BA the binding of silver to BA at pH 3 was found to decrease as shown in the two curves of Fig. 3. The amount of silver binding eliminated at the higher concentrations is very nearly 1 mole per mole BA. In another experiment of similar nature, but at pH 6 instead of pH 3, amounts of IA varying from zero to 10.8 moles per mole of BA were used to treat the protein. The amounts of silver bound per mole of the treated protein at pH 6 were determined at two different silver ion concentrations. The results of this experiment are given in Fig. 4 in which the amount of IA used to produce this reduction is the abscissa. Treatment with IA eliminated a maximum of 1 mole of silver binding at both silver ion concentrations.

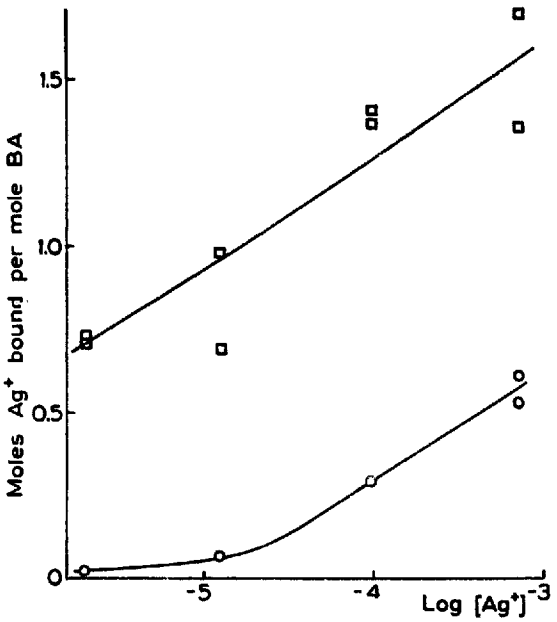


Fig. 3. Effect of IA treatment (6 moles IA per mole BA) on the binding of silver ion to Pentex BA at pH 3.0 (0.05 M citrate buffer). □—□, untreated; O—O, treated with IA.

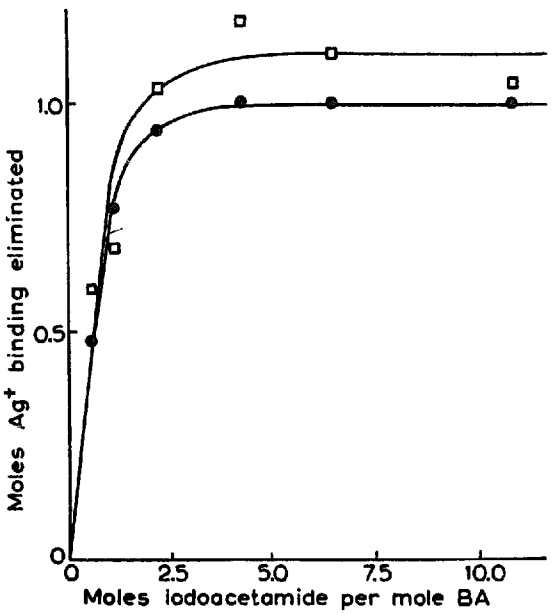


Fig. 4. The effect of IA treatment upon the binding of silver ion to Armour BA at pH 6 (0.05 M citrate buffer). □—□, 10^{-5} M Ag^+ and ●—●, $2 \cdot 10^{-6}$ M Ag^+.

Studies using ^{14}C-labelled IA

After treatment of BA with IA labelled with ^{14}C in the carboxyl group the protein was hydrolyzed and a search made by ion-exchange chromatography for the amino acid derivatives which would result from the reaction with the sulfhydryl group of the cysteine residue, CMC. Ion-exchange chromatography was performed on Dowex-1 in the formate form using a system to separate dicarboxylic amino acids, but not neutral or positively charged amino acids. The results of two such runs are presented in Table II. Recovery experiments in which amido-CM-cysteine and CMC were added to the protein before hydrolysis indicated 84% recovery of the added material in the form of CMC so that hydrolysis conditions destroyed some material.

The radioactivity appearing in Fractions A through F, especially Fractions A and D, are probably the result of the reaction of IA with the ε-amino group of lysine, the thioether group of methionine and imidazole group of histidine. That iodoacetic acid can react with these groups has been established in the work upon RNAase by GUNDLACH, STEIN AND MOORE[7].

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN HYDROLYSATES OF BA (ARMOUR LOT No. 666) TREATED WITH ^{14}C-LABELLED IA

Expt.		*5*	*6*
Titre in amperometric titration		0.76	0.76
Type of treatment		6.1 moles IA/mole BA for 36 h in phosphate buffer (pH 8)	10 moles IA/mole BA for 18 h in 8 M urea; 0.1 M Tris buffer (pH 8)
Moles activity/mole BA after dialysis		1.85	0.86
Moles activity/mole BA after hydrolysis		1.33	0.71
Moles activity/mole BA in each fraction in order of appearance in chromatogram	A	0.31	0.16
	B	0.04	
	C	0.05	0.03
	D	0.22	0.05
	E	0.07	0.01
	F	0.05	0.04
	G	0.60*	0.37*
	H		0.01
	I		0.05
Total all fractions		1.34	0.71

* This fraction contained CMC as its only component as determined by filter-paper chromatography of it.

DISCUSSION

BA when titrated amperometrically with silver ion in an amine or ammonia buffer generally gives a value of approx. 0.7 mole of sulfhydryl per mole BA (see ref. 3). KOLTHOFF, STRICKS AND MORREN[8] reported a similar value using mercuric ion. BA has been shown to contain both mercaptalbumin and a non-sulfhydryl-containing dimer[9,10].

In the above amperometric titration procedures the calculated free metal ion concentration is very low. For example in one of the procedures where a 0.3 M NH_3–NH_4NO_3 buffer (pH 10) is used with silver ion as the reagent to titrate the sulfhydryl group—the maximum free silver ion as calculated from the silver ammonia dissociation constant is approx. 10^{-11} M. AMBROSE, KISTIAKOWSKY AND KRIDLE[11] in studies on the inhibition of urease by silver ion found that inhibition of the enzyme began at a concentration of 10^{-11} M silver ion, inhibition increasing with increasing silver ion concentration. Inhibition was assumed to be due to argentation of the sulfhydryl group.

It was expected from the studies cited just above that the binding of silver to the protein sulfhydryl group would begin near 10^{-11} M silver ion, and would reach a plateau at about 0.7 mole of silver bound per mole BA; it could be expected to rise higher than the plateau value only at relatively high concentrations of silver ion, where cation binding of a non-specific nature would occur. The results shown in Fig. 1 indicated there was no appreciable binding of silver ion below 10^{-7} M silver

ion at pH 6, or, very likely, at pH 10, too; the amount of silver bound rose in a nearly linear fashion, thereafter, when plotted on a logarithmic scale. At no portion of the silver binding spectrum was there a plateau value reached in the vicinity of 0.7–1.0 mole of silver bound per mole of BA. In addition Expt. 91 of Fig. 1 suggests that the binding of silver ion was reversible at pH 6.

The results at pH 10 in borate buffer presented in Fig. 1 suggest that pH is not the significant factor in the reaction of silver with sulfhydryl under the conditions of the amperometric titration, namely in ammonia buffer. The results shown in Table I, part A, indicate that amounts of silver were bound to BA by equilibrium dialysis very similar to the titre of silver in the amperometric titration when dialysis was carried out in the presence of ammonia buffer. The results of part B of Table I suggest that the action of ammonia is irreversible.

The results presented in Fig. 2 indicated that denaturation of the protein by urea decreased the amount of silver bound, rather than increasing it, as would be expected if the sulfhydryl were only physically "masked". This suggests that the action of ammonia is not the same as a denaturing agent. A similar effect of urea was noted in Expt. 6 of Table II where less ^{14}C-labelled IA than normal reacted with BA in the presence of conc. urea solution. This result is opposite to that found by KLOTZ and co-workers[12] in a similar experiment with the protein hemerythrin. Besides being another protein, perhaps the difference in results is due to the fact that the titration of hemerythrin with silver ion was performed in the presence of an amine buffer.

Of the apparently large number of sites available for the binding of silver ion to the BA molecule at silver ion concentrations greater than 10^{-7} M, one site was found to be different than the rest in that it was reactive with IA. The results shown in Figs. 3 and 4 indicate that silver ion reacts with this site to the extent of 1 mole per mole of BA. Treatment of BA with 3–10 moles IA per mole BA eliminated 1 mole of silver binding at two different silver ion concentrations at pH 6. A very similar result was obtained at pH 3 as shown in Fig. 3 indicating pH independence of the binding to this 1 sulfhydryl-like site.

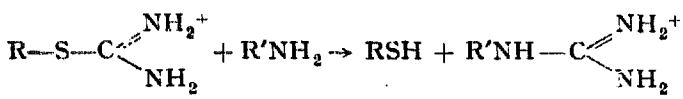
The foregoing equilibrium-dialysis studies clearly indicate that there is 1 group in the BA molecule which reacts with IA and which binds silver ion only at relatively high concentrations where binding to other groups takes place as well. That there is considerable certainty that this group is the sulfhydryl group in some form may be seen from the experiments in which BA treated with ^{14}C-labelled IA was hydrolyzed and the hydrolysis products examined by ion-exchange chromatography. CMC is the compound expected to appear in the protein hydrolysate from the reaction between a cysteine residue of the protein and IA. As can be seen in Table II this compound was identified as the product present in the largest radioactive fraction.

These observations upon BA suggest some conclusions as to the nature of the sulfhydryl group in this protein. The results of the equilibrium-dialysis studies indicate that the concentration of silver ion required for reaction with the sulfhydryl group is much greater than would be expected from the estimated equilibrium constant for silver–sulfhydryl group interaction. The classical concept of the physically masked sulfhydryl group existing free, but largely inaccessible, within the folds of the protein seems not to apply. A reversible association of silver ion with a group of low affinity is involved and relatively high concentrations of free silver ion are required to force the equilibrium in the direction of argentation except in the presence of ammonia.

Ammonia may serve to disrupt this group so that free sulfhydryl is available for reaction.

LINDERSTRÖM-LANG AND JACOBSEN[13] using the model compound 2-methylthiazoline demonstrated that reactive sulfhydryl was released from this compound by reaction with ammonium ion. They found, too, that this compound manifested many of the characteristics of a "masked" sulfhydryl group in its ability to release sulfhydryl when treated with appropriate reagents.

When BA was treated with ammonium ion, however, no difference was found in the pattern of its binding of silver ion. It was only in the presence of ammonia that the protein bound silver at very low free silver ion concentrations. As an actual link existing in protein, therefore, a possibility in analogy to the thiazoline chemistry elucidated by LINDERSTRÖM-LANG AND JACOBSEN[13] is that of the 2-aminothiazoline or isothiuronium-type compounds. The chemistry of these compounds has more recently been investigated, and offers a plausible mechanism in model-type compounds for reactions observed in the above studies on BA. The general type of these reactions is the following:



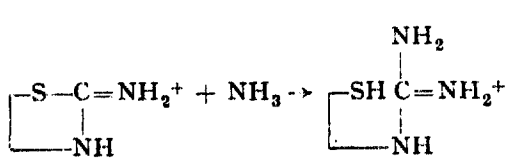
(1)

KHYM, SHAPIRA AND DOHERTY[14] investigated the chemistry of amino ethylisothiuronium salts and found reactions of this general type to occur; an intramolecular transfer of the amidine group occurred to produce mercaptoethylguanidine and 2-aminothiazoline at pH 7 as products. WALKER AND WALKER[15] postulated a very similar mechanism to occur in the enzyme transamidinase. In this enzyme it is thought that the amidine group is reversibly transferred from arginine or guanidine acetate to an acceptor such as ornithine or glycine. They postulated an S-enzyme isothiourea intermediate with transfer of the amidine group to an acceptor similar to Reaction 1 above.

MAEKAWA AND LIENER[16] used the reaction of *S*-methylglucosyl isothiourea with amino groups to produce the enzymically active glucosyl-amidyl derivative of trypsin. This reaction with protein was earlier studied by MICHEEL AND HEROLD[17].

These studies indicate that sulfur bound in isothiuronium-type compounds is released as sulfhydryl upon aminolysis.

The greatly diminished reactivity of the BA "sulfhydryl group" with silver ion and its increased reactivity upon exposure to ammonia suggest a type of chemical reaction similar to that of the isothiuronium compounds. Thus, aminolysis of an isothiuronium bond in BA would liberate the sulfhydryl group of cysteine residue and regenerate the guanidine moiety of an arginine residue as follows:



Such an hypothesis offers an explanation in accord with the results obtained in the experiments described.

REFERENCES

1 I. M. KOLTHOFF AND W. E. HARRIS, *Ind. Eng. Chem.*, 18 (1946) 161.
2 R. H. A. CRAWLEY, *Chem. Ind. London*, (1953) 1205.
3 R. E. BENESCH, H. A. LARDY AND R. J. BENESCH, *J. Biol. Chem.*, 216 (1955) 663.
4 T. R. HUGHES AND I. M. KLOTZ, *Methods of Biochemical Analysis*, Vol. 3, Interscience, New York, 1956, p. 265.
5 L. MICHAELIS AND M. P. SHUBERT, *J. Biol. Chem.*, 106 (1934) 340.
6 I. M. KLOTZ, *Cold Spring Harbor Symp. Quant. Biol.*, 14 (1949) 23, 97.
7 H. G. GUNDLACH, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 234 (1959) 1754.
8 I. M. KOLTHOFF, W. STRICKS AND L. MORREN, *Anal. Chem.*, 26 (1954) 366.
9 T. P. KING, D. A. YPHANTIS AND L. C. CRAIG, *J. Am. Chem. Soc.*, 82 (1960) 3350.
10 A. TISELIUS, S. HJERTEN AND O. LEVIN, *Arch. Biochem. Biophys.*, 65 (1956) 132.
11 J. F. AMBROSE, G. B. KISTIAKOWSKY AND A. G. KRIDLE, *J. Am. Chem. Soc.*, 73 (1951) 1232.
12 I. M. KLOTZ, T. A. KLOTZ AND H. A. FIESS, *Arch. Biochem. Biophys.*, 68 (1957) 284.
13 K. LINDERSTRÖM-LANG AND C. F. JACOBSEN, *J. Biol. Chem.*, 137 (1941) 443.
14 J. X. KHYM, R. SHAPIRA AND D. G. DOHERTY, *J. Am. Chem. Soc.*, 79 (1957) 5667.
15 J. B. WALKER AND M. S. WALKER, *Arch. Biochem. Biophys.*, 86 (1960) 80.
16 K. MAEKAWA AND I. E. LIENER, *Arch. Biochem. Biophys.*, 91 (1960) 108.
17 F. MICHEEL AND B. HEROLD, *Z. Physiol. Chem.*, 293 (1953) 187.