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Decrease in Sulfhydryl Titer of Serum Albumin[1]

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The sulfhydryl content of human and bovine serum albumin is always considerably below 1 mole SH per mole. We find that the sulfhydryl titer, as determined by titration with methylmercuric iodide, decreases over a period of days, and the rate of decrease is greater in acid or alkaline solutions than at neutral *p*H, where it is very slow. The rate of decrease in acid solution is maximum at *p*H 3 and is greater in HCl than in HNO_3. The amount of dimer formed in acid solutions is inadequate to account for the loss of sulfhydryl through disulfide formation between molecules. Deoxygenation of the solutions has little effect on the reaction. Attempts to reverse the reaction by addition of excess methylmercuric ion were unsuccessful. Possible reasons for the absence of sulfhydryl titer in part of the serum albumin are discussed.

Although the fraction of serum albumin showing a sulfhydryl group (and therefore called mercaptalbumin) is ordinarily about two-thirds,[2] this fraction is often smaller in albumin that has stood a long time.[3,4] We have investigated the effect of some conditions on the rate of decrease in sulfhydryl titer.

Experimental

The human serum albumin was from Batch 1200 processed by Squibb and donated by Dr. J. N. Ashworth of the American Red Cross. The bovine serum albumin was Armour's fraction V. Its solution had a darker reddish-brown color than the human albumin. The methylmercuric iodide (m.p. 144–146°) was prepared by the method of Maynard.[5] Its toluene solution was stored in the refrigerator. The methylmercuric nitrate solution was prepared by metathesis of equivalents of aqueous silver nitrate and methylmercuric bromide[6] dissolved in a small amount of alcohol, followed by filtering off the precipitate of silver bromide.

The sulfhydryl analysis was a slight modification of a method of Hughes.[2] To a measured volume of albumin solution containing about one micromole of sulfhydryl was added phosphate buffer and enough acid or base to bring the *p*H to between 7.0 and 7.5. A measured excess of millimolar methylmercuric iodide in toluene was added. Then the two phases were equilibrated by mechanical rotation for an hour or longer. An aliquot (approximately 0.2 ml.) of the toluene layer (which at equilibrium contains practically all the excess methylmercuric iodide) was added to approximately half a milliliter of 50–50 pyridine–glacial acetic acid plus a drop of 0.1 *M* ethylenediaminetetraacetate to complex interfering metals. This was then titrated with 0.2 millimolar dithizone in chloroform or carbon tetrachloride (from a needle valve buret[7] to eliminate stopcock grease) until the first excess of dithizone imparted a green color to the solution. Since the dithizone is subject to slow air oxidation, it was standardized against 0.2 millimolar methylmercuric iodide.

This method agreed very closely with the somewhat less precise single-phase titration with methylmercuric nitrate using nitroprusside indicator[8] and with an amperometric mercuric chloride titration with the polarograph.[9]

The *p*H of albumin solutions was measured after dilution to 1 or 2% protein.

In order to ensure complete permeation by nitrogen without bubbling, a deoxygenator was devised using a syringe as a bearing. To accommodate a test-tube containing the albumin solution, the shortened barrel of a syringe was fitted with a rubber sleeve (made by boring a hole in a stopper). All this was mechanically rotated about a stationary cylinder made by cutting off both ends of the syringe plunger so that a nitrogen inlet tube could go through a two-hole stopper at the top of the cylinder and almost touch the solution at the bottom. After deoxygenation in this apparatus, the test-tube containing the albumin solution was transferred to a two-hole stopper for final deoxygenation and storage.

To show that bacterial contamination was not responsible for the loss in sulfhydryl, two samples of albumin showing a typical loss of sulfhydryl after 12 days at *p*H 3.5 were plated on a culture medium by Dr. Robert J. Fitzgerald of the National Institute of Dental Research. One sample contained 220 organisms per ml. and the other no growth.

Results

Figure 1 shows typical decreases in sulfhydryl titer of human serum and bovine albumin samples brought to various *p*H's with HCl or NaOH and stored in the refrigerator (approx. 5°). After a small initial drop in sulfhydryl content on addition of acid or base, the decrease thereafter follows first-order kinetics sufficiently well that we can plot a first-order rate constant as a function of *p*H[10] (Fig. 2). This figure also shows that the rate of de-

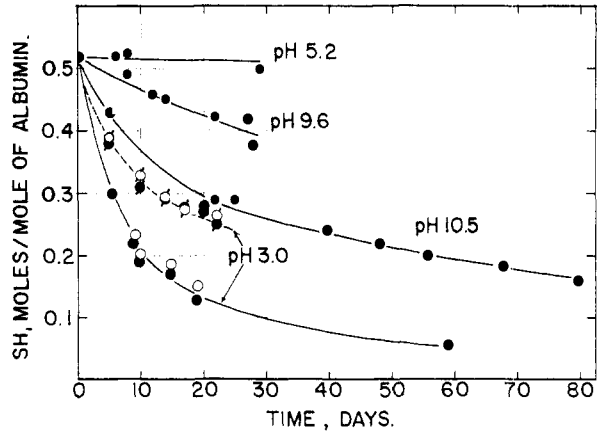

Fig. 1.—Decrease in sulfhydryl titer of serum albumin in HCl or NaOH: filled circles, open to the air; open circles, deoxygenated; solid curves, human serum albumin; dashed curve, bovine serum albumin.

(1) Presented at the 130th meeting of the American Chemical Society at Atlantic City, New Jersey, in September, 1956.

(2) W. L. Hughes, Jr., *Cold Spring Harbor Symposia on Quantitative Biology*, **80**, XIV (1950).

(3) R. Benesch and R. E. Benesch quoted by C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6416 (1955).

(4) W. L. Hughes, Jr., personal communication.

(5) J. L. Maynard, THIS JOURNAL, **54**, 2108 (1932).

(6) We wish to thank Dr. W. L. Hughes, Jr., for the methylmercuric bromide.

(7) Emil Greiner Co., 20–26 N. Moore St., N. Y. 13, N. Y.

(8) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5059 (1953).

(9) H. A. Saroff and H. J. Mark, *ibid.*, **75**, 1420 (1953).

(10) The maximum at *p*H 3 is reminiscent of that observed by J. T. Yang and J. F. Foster, *ibid.*, **76**, 1588 (1954), in the *p*H dependence of optical rotation and viscosity.

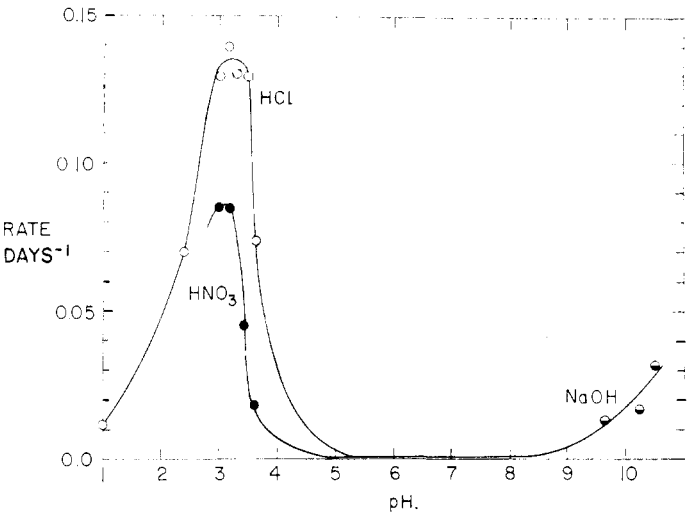

Fig. 2.—Rate of decrease of sulfhydryl titer of human serum albumin as a function of *p*H.

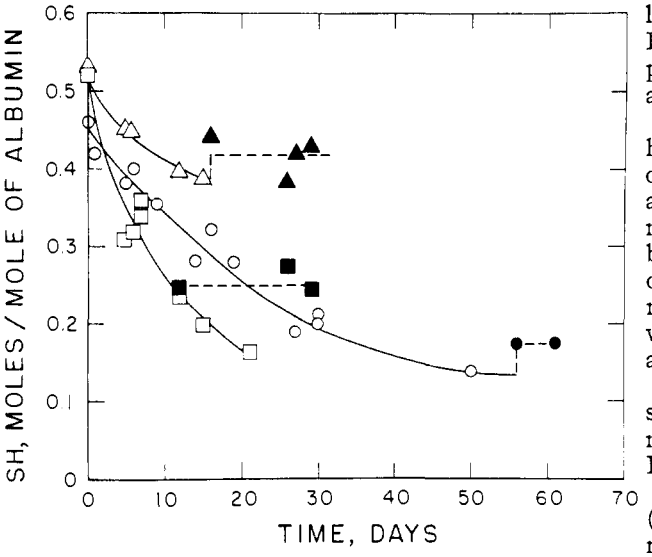

Fig. 3.—Attempts to reverse decrease in sulfhydryl titer of human serum albumin: △, *p*H 3.3 HNO_3; ○, *p*H 3.2 HNO_3; □, *p*H 3.3 HCl; dashed lines and filled symbols, after addition of 0.60 mole CH_3HgNO_3 per mole of albumin.

crease is slower when nitric acid rather than hydrochloric is used to lower the *p*H.

Although the rate of decrease at neutral *p*H's is too slow to show change over a period of days, evidence for such a reaction was obtained by titration of solutions of human serum albumin stored in sterile bottles for approximately five years. These showed negligible SH content (less than 0.05 mole SH per mole albumin).

TABLE I

	Moles SH per mole alb.	% Dimer	Decrease in SH, moles per mole alb.
*p*H 5	0.50	8	
Isoelectric stored 5 years	.05	7	0.50
*p*H 1.1, 75 days	.25	27	.27
*p*H 3.5, 6 days	.30	15	.22
*p*H 10.2, 35 days	.35	7	.17
*p*H 10.5, 39 days	.22	18	.30

To determine whether disulfide bonds had been formed between molecules, ultracentrifuge runs[11] were made on these 5-year old solutions and showed that the amount of albumin with sedimentation constant corresponding to the dimer was negligible (Table I). Ultracentrifuge runs on other solutions did show some increase in a component with sedimentation constant of the dimer but not sufficient except at *p*H 1.1 to account for all the loss in SH.

All runs after 48 min. in 0.1 *M* $NaNO_3$ at 250,000 × *g* on Spinco ultracentrifuge.

The effect of oxygen on the rate was found to be very small by experiments in which the albumin was made acidic or basic and a portion immediately deoxygenated. The rate of decrease of sulfhydryl was almost as great in the deoxygenated portion as in the portion stored in air (Fig. 1).

The rate of decrease in SH titer of a sample of bovine serum albumin was considerably less than that of human serum albumin (Fig. 1). Bovine serum albumin in which about 57 groups per mole had been guanidinated[12] showed only about 0.1 mole SH per mole of albumin.

In an attempt to reverse the decrease in sulfhydryl, a measured excess of an aqueous solution of methylmercuric nitrate was added to several albumin samples. At intervals, aliquots were removed and titrated by the usual two-phase equilibration method after the addition to the aliquot of potassium iodide solution equivalent to the methylmercuric nitrate present. Although there was apparently a slight increase initially, little if any further change occurred (Fig. 3).

To determine whether urea would increase the sulfhydryl titer we performed an experiment with methylmercuric iodide similar to that of Benesch, Lardy and Benesch[13] with silver.

Using 0.1 *M* tris-hydroxymethylaminomethane (*p*H 7.4) as buffer, we dissolved samples of albumin in water and in 8.0 *M* deionized urea and equilibrated each with an excess of methylmercuric iodide in toluene. Immediate titration of the excess methylmercuric iodide yielded approximately the same value (0.55 mole SH per mole of albumin) in both cases. The SH titer of the albumin in urea decreased from 0.42 to 0.28 mole per mole of albumin in one day. Since the SH titer of the albumin at this *p*H would remain constant for at least 60 days in the absence of urea, the denaturing agent increased the rate at which the sulfhydryl decreased.

Discussion

Since no differences (other than sulfhydryl titer) have been noted between the total albumin and the mercaptalbumin, the question arises whether the non-mercaptalbumin may not have the same amino acid sequence as the mercaptalbumin[14,15] but have

(11) We wish to thank Emil Adamik for the ultracentrifuge runs.

(12) W. L. Hughes, Jr., H. A. Saroff and A. L. Carney, THIS JOURNAL, **71**, 2476 (1949).

(13) R. E. Benesch, H. A. Lardy and R. Benesch, *J. Biol. Chem.*, **216**, 663 (1955).

(14) W. L. Hughes, Jr., in "The Proteins," Vol. II, edited by H.

the sulfhydryl unavailable to reagents, either because of "masking" or because of chemical reaction. Although an amperometric silver titration with a rotating platinum electrode showing an increase in the presence of 8 *M* urea[13] suggests that an inaccessible sulfhydryl becomes available on denaturation, titration of albumin with methylmercuric iodide does not show any increase in 8 *M* urea or in 3 *M* guanidine hydrochloride.[17] In fact, our experiments show an increase in the rate of loss of sulfhydryl on denaturation with urea.

M. J. Hunter[18] and Katchalski, Benjamin and Gross[19] have shown that reduction increases the sulfhydryl content of albumin under conditions where there is no increase in sulfhydryl content of mercaptalbumin suggesting that the sulfhydryl in non-mercaptalbumin has been oxidized, but it is difficult to write a reasonable formula for the oxidized sulfhydryl. Although there is no proof that the reaction causing loss of sulfhydryl in acidic or basic solution is the reaction responsible for the low sulfhydryl content of ordinary albumin, it is worth noting that the former reaction is very little affected by molecular oxygen.

In view of the uncertainties in our present knowledge of the nature of non-mercaptalbumin, we mention another possibility for reaction of the sulfhydryl group. Linderstrøm-Lang and Jacobsen[20] suggested that the following reactions might occur in proteins (Fig. 4).

The analogous reaction of the hydroxyl group of serine or threonine to form the ester has been shown to occur in lysozyme after several hours in anhydrous formic acid[21] and in insulin on treatment with 0.03 *N* 85% (v./v.) ethanolic HCl.[22] Very little, if any, ester is formed in bovine serum albumin solutions kept at *p*H 3.5 or *p*H 2.2 for two weeks, for Van Slyke determinations of these agreed within 0 ± 2 free amino groups[23] with those of albumin kept at neutral *p*H. These findings do not exclude the possibility of oxazoline ring formation in serum albumin. There is evidence from absorption spectra and other data for the thiazoline ring form in bacitracin A[24,25] and coenzyme A,[26] although con-

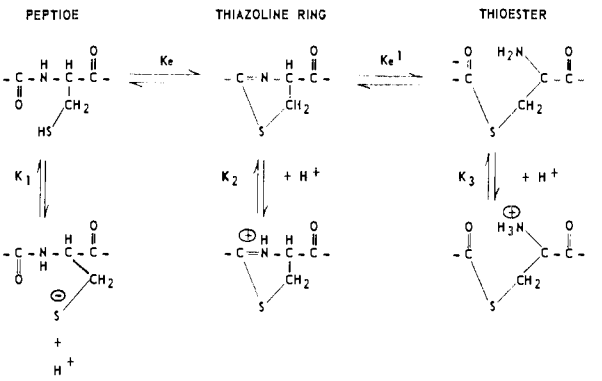

Fig. 4.—Hypothetical SH reactions.

$$\text{Ratio} = \frac{\text{Concn. of R—SH and R—S}^- \text{ species}}{\text{Concn. of all other species}}$$

$$\text{if } K_e > K_e'; \quad \frac{\text{Ratio}}{K_e} = \frac{1 + 1/K_1H^+}{1 + K_2H^+}$$

version from one form to the other has not been demonstrated.

The many other absorbing groups in serum albumin make it difficult to obtain evidence from spectra for or against a thiazoline ring, so we can consider our kinetic data as possible evidence. From Fig. 4 it may be deduced that for a given K_e the fraction of sulfhydryl form at equilibrium will be larger at higher *p*H values. If the reactions in Fig. 4 account for the loss of sulfhydryl titer of serum albumin in both acid and alkaline solution, then the equilibrium must greatly favor the thiazoline ring or thioester forms, for very little sulfhydryl is found on standing in alkaline solution. The reaction mechanism may, however, be different in alkaline solution.

Even in acid solution the lack of reversal to a high sulfhydryl titer in the presence of a large excess of methylmercuric ion shows that a reversible equilibrium is not attained. This, however, may not be a serious objection to the thiazoline ring hypothesis in serum albumin, for bacitracin A does not readily reverse to the sulfhydryl form with excess of sulfhydryl reagents.

Although loss of sulfhydryl does occur in the *p*H range of 4 to 10 where the albumin is not denatured, the addition of denaturing agents such as acid, alkali or urea greatly increases the rate of loss of sulfhydryl. The increased rate of loss of sulfhydryl might be a primary manifestation of the denaturation, or it might be any secondary reaction, such as thiazoline ring formation, facilitated by the increased flexibility of the unfolded albumin.

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Neurath and K. Bailey, Academic Press, New York, N. Y., Part B, p. 690.

(15) Indirect support for this view is our finding that two components of albumin separated by Sober, Gutter, Wyckoff and Peterson[16] on anion-exchange cellulose columns had identical sulfhydryl titers.

(16) H. A. Sober, F. J. Gutter, M. M. Wyckoff and E. A. Peterson, THIS JOURNAL, **78**, 756 (1956).

(17) W. L. Hughes, Jr., personal communication.

(18) M. J. Hunter, quoted in reference 14.

(19) E. Katchalski, G. S. Benjamin and Violet Gross, THIS JOURNAL, **79**, 4096 (1957).

(20) K. Linderstrøm-Lang and C. F. Jacobsen, *J. Biol. Chem.*, **137**, 443 (1941).

(21) L. Josefsson and P. Edman, *Biochim. et Biophys. Acta*, **25**, 614 (1957).

(22) A. C. Chibnall and M. W. Rees, "The Chemical Structure of Proteins," Ciba Foundation Symposium, edited by G. E. W. Wolstenholme and M. P. Cameron, J. & A. Churchill Ltd., London, 1953.

(23) H. A. Saroff and A. Middleton, unpublished results.

(24) J. R. Weisiger, W. Hausmann and L. C. Craig, THIS JOURNAL, **77**, 3123 (1955).

(25) G. G. F. Newton and E. P. Abraham, *Biochem. J.*, **53**, 604 (1953).

(26) R. E. Basford and F. M. Huennekens, THIS JOURNAL, **77**, 3878 (1953).