treated with 302 mg. of $FeCl_3 \cdot 6H_2O$ at room temperature in an atmosphere of nitrogen. After a few minutes a brilliant red color was observed which appeared to be stable. After 1 hr., water was added and the solution extracted with ether. The combined ether extracts were washed with water, dried over sodium sulfate and evaporated to dryness. Even after chromatography on Alumina, the red colored oxidation product remained amorphous, m.p. 185–187° (under dec.).

Anal. Calcd. for $C_{16}H_{10}O_5$ (282.2): C, 68.09; H, 3.54. Found: C, 67.94; H, 3.69.

The ultraviolet spectrum revealed absorption at λ_{max} 209, 245 and 282 mμ.

Degradation with Alkali.—Fifty mg. of the original product was fused with 10 pellets of KOH for 1 hr. at 200° in an atmosphere of nitrogen. The brown melt was taken up in water and extracted with ether. From the alkaline ether extract 25 mg. of starting material was recovered in crystalline form, m.p. 117–118°. The alkaline aqueous solution was acidified with dilute HCl and continuously extracted with ether. The dried and evaporated ether extracts were subjected to high vacuum sublimation at 1×10^{-4} mm. in a Willstätter tube. At an air-bath temperature of 120–125°, a colorless crystallized sublimation product was found in the first receiver. After recrystallization from ether–petroleum ether, 4 mg. of prismatic needles was obtained, m.p. 190–191° (Berl, uncor.).

The infrared analysis offered conclusive evidence that the alkaline degradation product was a non-substituted aromatic carboxylic acid. This is substantiated by the broad absorption extending from 3400 to 2200 cm.$^{-1}$ with submaxima at 2670 and 2445 cm.$^{-1}$. The absence of any absorption from 3400–4000 cm.$^{-1}$ provides evidence that the acid does not contain a phenolic or alcoholic hydroxyl group.

PHILADELPHIA, PA.

[CONTRIBUTION NO. 1447 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

On the Aggregation of Bovine Serum Albumin in Acid Solutions[1,2]

BY P. BRO,[3] S. J. SINGER AND J. M. STURTEVANT

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Bovine serum albumin forms aggregates in 0.10 *M* NaCl-HCl solutions below *p*H 3.4. The aggregation reaction appears to be reversible with respect to *p*H, but the rate of the reaction in both directions is subject to erratic fluctuations. The reaction is inhibited by excess mercuric ion. The aggregates can be converted to monomer on an ion exchange column in the presence of thioglycolate. The experimental evidence available is consistent with the hypothesis that the reaction is a thiol–disulfide exchange.

Introduction

It has been observed by several investigators that bovine serum albumin (BSA) may aggregate in acid solutions. Reichmann and Charlwood,[4] observed aggregates at *p*H 1.9 in the absence of added salts. In 0.10 *M* KCl solutions aggregates did not appear to form, whereas they did form in 0.50 *M* KCl solutions. Saroff, Loeb and Scheraga[5] found that aggregates appeared in 0.10 *M* NaCl and sodium acetate solutions of BSA. The aggregation phenomenon was investigated in some detail by Kronman, Stern and Timasheff[6] who attempted to establish the conditions under which the aggregation reaction occurs. Their findings were inconclusive in that no clearly defined physical and chemical factors could be isolated which were responsible for the formation of aggregates. Aggregates also were observed by the present authors,[7] who reported on the sedimentation behavior, and made the observation that the aggregate appeared to undergo a reversible swelling similar to that observed with monomeric BSA.

During a series of calorimetric studies on serum albumin, the question arose as to the effects which the aggregation reaction might have on the thermochemical behavior of the protein at low *p*H. In order to answer that question it was necessary to learn more about the chemical nature of the aggregates and to devise a method for changing the aggregates back to monomer.

Experimental

Crystalline BSA was purchased from Armour and Co. (Lot G-4302) and from Pentex, Inc. (Lots A-1201 and B-12016 P). Crystalline bovine mercaptalbumin (BMA) was prepared by way of its mercury dimer by the method described by Dintzis.[8] Analytical grade reagents were used throughout. The investigations were carried out with approximately 1% protein in NaCl-HCl solutions 0.10 *M* in Cl^-, unless otherwise stated, at approximately 25°. The protein concentration of the various solutions was measured with a Phoenix B-S differential refractometer calibrated with albumin solutions of known concentration as determined by the micro-Kjeldahl method. The nitrogen content of the albumin was taken to be 16.07%. The experimental results are reported in terms of the *p*H of the solutions as measured with a Beckman model G *p*H-meter at 25°, the *p*H-meter being standardized with a 0.10 *M* ionic strength acetate buffer having a *p*H of 4.65.[9]

The extent of the aggregation reaction was measured with a Spinco Model E ultracentrifuge operated at 59,780 r.p.m. Ultracentrifuge cells with plastic centerpieces were used. In analyzing the sedimentation patterns, it was assumed that the refractive increments of the various aggregates are the same as that of the monomer, and the areas under the partially resolved peaks of the sedimentation patterns were divided symmetrically. The ratios of the areas were taken as the ratios of the concentrations of the various components. The usual correction for the dilution effect was introduced. The errors in the observed areas due to the Ogston–Johnston[10] effect were within the experimental error. On the basis of the analysis of 24 different sedimentation patterns, the standard deviation in the determination of the aggregate to monomer ratio was found to be 0.04 and to be independent of the value of the ratio.

The aggregrate under investigation here, al-

(1) From a dissertation submitted by P. Bro in partial fulfillment of the requirements for the Ph.D. degree, June, 1956.

(2) This research was aided by grants from the National Science Foundation (G 179) and the United States Public Health Service (RG 3996 C).

(3) General Electric Co. Fellow, 1954–1956.

(4) M. E. Reichmann and P. A. Charlwood, *Can. J. Chem.*, **32**, 1092 (1954).

(5) H. A. Saroff, G. I. Loeb and H. A. Scheraga, THIS JOURNAL, **77**, 2908 (1955).

(6) M. J. Kronman, M. D. Stern and S. N. Timasheff, *J. Phys. Chem.*, **60**, 829 (1956).

(7) P. Bro, S. J. Singer and J. M. Sturtevant, THIS JOURNAL, **77**, 4924 (1955).

(8) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

(9) R. G. Bates, *Chem. Revs.*, **42**, 1 (1948).

(10) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

though apparently dimeric in character, is not to be confused with the mercury dimer[11] formed from mercaptalbumin, nor with the disulfide dimer of mercaptalbumin formed by oxidation.[12] Several differences between the low *p*H dimer and the others will appear in the results to be discussed below.

Results

The albumin samples as purchased contained approximately 5% of a heavy component (Fig. 1a). The low *p*H aggregation under study gave much higher concentrations of heavy components (Fig. 1b), ranging as high as 65% with the Armour BSA and 87% with BMA. Although at least two heavy components were observed in acid BSA solutions, our attention was concentrated on the smallest of these since it was more readily available for quantitative studies. The comparison of sedimentation constants of the various aggregates with that of the mercury dimer of BMA, given in Table I, indicates that the smallest aggregate is a dimer.

TABLE I

SEDIMENTATION CONSTANTS AT *p*H 3.0 OF BSA AND ITS AGGREGATES AND OF THE MERCURY DIMER OF BMA

Substance	$S_{w,20}$	Substance	$S_{w,20}$
BSA	3.06	Second aggregate	5.12
First aggregate	3.91	Hg dimer of BMA	3.97

The *p*H Dependence of the Aggregation and its Reversal.—Solutions of BSA were adjusted to several different *p*H values and examined in the ultracentrifuge after varying intervals at room temperature. The results, summarized in Table II, indicate that a significant increase in the concentration of dimer occurs at *p*H values below 3.40, a conclusion substantiated by numerous additional experiments. This observation is in disagreement with the report by Reichmann and Charlwood[4] that no aggregation takes place in 0.10 *M* KCl solutions at low *p*H. The data in the table illustrate the erratic rate with which the reaction takes place. Experiments performed with exclusion of oxygen showed that oxygen has no significant effect on the rate.

TABLE III

THE REVERSAL OF THE AGGREGATION REACTION AT HIGH *p*H

*p*H of aggregation	Age, hr.	Aggregate, %	*p*H of reversal	Age, hr.	Aggregate, %
2.97	26	34	7.15	2	5
2.97	49	34	3.61	2	5
2.97	69	34	3.31	2	31
2.97	74	34	3.41	3	28
3.11	..	65	5.17	24	59

Comparison of the Aggregate with the Mercury Dimer of BMA.—A sample of Pentex crystalline BSA was analyzed spectrographically and found to contain the following metals[13]: copper, 20 ± 3 p.p.m.; tin, less than 2 p.p.m.; zinc, 20 ± 3 p.p.m.; lead, less than 2 p.p.m. A sample of BSA solution containing 30% of the dimeric aggregate was found to contain very nearly the same metallic contamination on a dry protein basis. These amounts of metallic ions, if fully utilized in forming dimers analogous to the mercury dimer of BMA, could account for no more than 9% of dimer. It is therefore evident that the dimer present was largely of some other type.

Further evidence for this conclusion was obtained from a comparison of various properties of the mercury dimer of BMA with those of the aggregate. Previous study of the mercury dimer of mercaptalbumin[8,11,14] has been largely confined to *p*H values above 5.0. It was therefore necessary to investigate the stability at low *p*H of the mercury dimer formed at high *p*H and the stability at high *p*H of the mercury dimer formed at low *p*H. After this investigation was in progress, Kay and Edsall[15] published their quantitative studies which demonstrated the stability of the mercury dimer of BMA at low *p*H. Our qualitative results confirm their findings. BMA prepared by the method of Dintzis[8] was used in the experiments summarized in Table IV, at molar proportions of $HgCl_2$:BMA of 1:2. It is apparent that with respect to stability at high *p*H, even after preparation at low *p*H, the mercury dimer differs significantly from the low *p*H aggregate. As required by the dimerization equilibria[11,14]

$$2\text{BMA-SH} + HgCl_2 \rightleftarrows (\text{BMA-S})_2Hg + 2HCl \quad (1)(a)$$

$$(\text{BMA-S})_2Hg + HgCl_2 \rightleftarrows 2\text{BMA-SHgCl} \quad (b)$$

the mercury dimer was found to disappear rapidly on the addition of an excess of $HgCl_2$.

The series of experiments in Table V demonstrates that the mercury dimerization of BMA takes place also in the presence of BSA at low *p*H.

TABLE II

THE APPEARANCE OF DIMERIC AGGREGATE OF BSA AS A FUNCTION OF *p*H

*p*H	2.07	2.48	2.70	2.81	2.90	2.90	2.99	3.30	3.40	3.68
Age of soln., days	1	1	1	1	1	5	1	1	2	2
Dimer, %	15	18	9	40	24	34	47	48	10	5

To test the possibility that the absence of aggregates at *p*H values above 3.40 might be due to rate control rather than to equilibrium control, solutions in which aggregate had formed at a low *p*H were adjusted to a higher *p*H and after various lengths of time subjected to ultracentrifugation. The first four experiments listed in Table III indicate that the aggregation can be reversed by raising the *p*H, so that the reaction can properly be described as a low *p*H phenomenon. The last experiment in the table indicates that the reversal of aggregation, like the aggregation reaction itself, is erratic in nature.

(11) (a) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947); (b) W. L. Hughes, Jr., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 79 (1950).

(12) R. Straessle, THIS JOURNAL, **76**, 3138 (1954).

(13) Analyses performed by Ledoux and Co., Teaneck, N. J. In preparing the sample for spectrographic analysis, the elements Hg and As probably are lost.

(14) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

(15) C. M. Kay and J. T. Edsall, *Arch. Biochem. Biophys.*, **65**, 354 (1956).

TABLE IV

THE STABILITY OF THE MERCURY DIMER OF BMA AT VARIOUS VALUES OF *p*H

*p*H of prepn. of dimer	5.13	5.13	5.13	5.13	3.0	3.0	3.0
*p*H of aging	4.57	4.02	3.77	3.48	3.35	3.60	4.92
Age, days	2	2	2	3	2	2	2
Dimer, %	35	75	79	66	80	66	64

The protein used contained 57% BMA, so that maximum dimer formation should be observed with a mole ratio of Hg^{++} to BSA of 0.29.

TABLE V

FORMATION OF THE MERCURY DIMER OF BMA IN THE PRESENCE OF BSA AT *p*H 2.93

Hg^{++}/BSA mole ratio	0	0.22	0.54	0.75	1.60	2.70
Age, days	2	1	2	1	2	2
Dimer, %	5	34	14	8	6	5

Whereas excess $HgCl_2$ leads to complete and rapid replacement of the mercury dimer by BMA–SHgCl, only a very slow disappearance of the low *p*H aggregate is caused by excess $HgCl_2$. This is illustrated by the data in Table VI.

TABLE VI

ACTION OF EXCESS $HgCl_2$ ON THE LOW *p*H AGGREGATE

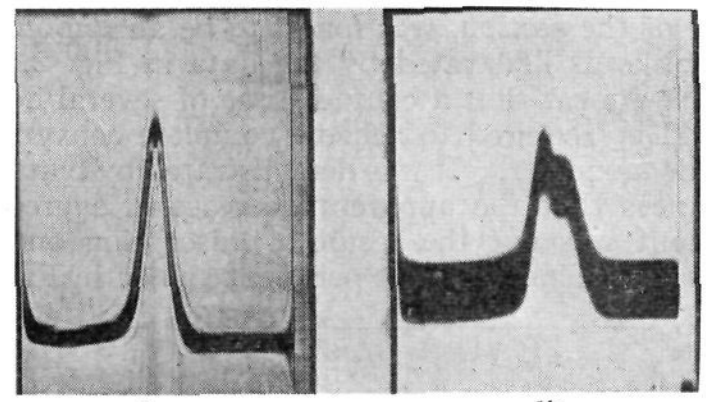
Hg^{++}/BSA mole ratio	0.00	0.52	0.52	1.57	1.57
*p*H	5.2	5.2	3.0	3.0	3.0
Age, days	1	1	2	3	8
Dimer, %	31	37	23	21	13

The small apparent increase in dimer at *p*H 5.2 may be due to the formation of some mercury dimer. It may be noted that the lack of *p*H reversal in the first 2 samples in the table is similar to what was observed with the last sample in Table III.

A series of experiments was performed to see whether the low *p*H aggregation is inhibited by a large excess of Hg^{++}. These experiments were performed about a year later than the other experiments described in this paper, and utilized different samples of proteins. With BSA and BMA at *p*H 3.0 in the absence of $HgCl_2$, with aging periods of 1 to 5 days, amounts of aggregate ranging up to 22% were observed. On the other hand, with Hg^{++}:SH ratios from 5 to 10, no aggregate formation was observed in any case. Although the experiments with no $HgCl_2$ present illustrated again the erratic character of the reaction, it may be concluded that excess $HgCl_2$ completely inhibits the low *p*H aggregation.

It is evident from the experiments detailed above that the low *p*H aggregate is of a type quite different from the mercury dimer of BMA. Nevertheless, there appears to be a direct connection between the presence of BMA and the low *p*H aggregation phenomenon. Samples of BSA which contain approximately 60% of BMA as shown by sulfhydryl titrations[8] have yielded a maximum of 65% of dimeric aggregate. On the other hand, a solution of BMA behaved as shown in Table VII, giving eventually nearly complete dimerization. No aggregation of BMA was observed at *p*H values above 3.4.

Furthermore, experiments were performed with



1a 1b

Fig. 1.—Ultracentrifuge diagrams of crystalline BSA; sedimentation proceeds to the left: (a) sample that did not aggregate, in 0.1 *M* NaCl–HCl, *p*H 2.91, after 126 minutes at 59,780 r.p.m.; (b) sample containing 59% dimer, in 0.1 *M* NaCl–HCl, *p*H 3.11, after 88 minutes at same speed.

a BSA preparation whose sulfhydryl groups were completely blocked by reaction with a derivative of iodoacetamide, N-(*p*-benzenearsonic acid)-iodoacetamide. This preparation, labeled BSA–S–R_1, has been described in other studies.[16] Analyses indicated that one atom of arsenic was attached per SH group originally present in the BSA sample, and all SH was reacted. In five experiments with BSA–S–R_1 aged from 3 to 5 days at *p*H 2.9, no aggregates were observed, which result implicates the free sulfhydryl group of BMA in the aggregation reaction.

TABLE VII

AGGREGATION OF BMA AT *p*H 3.0

Age, hr.	1	4	7	10
Dimer, %	11	21	29	87

The Effect of Reducing Agents on the Aggregate.—Straessle[12] has shown that the disulfide dimer of human mercaptalbumin, formed by oxidation of the mercury dimer by iodine, can be converted to the monomer by the reducing action of cysteine. It was therefore of interest to investigate the effect of reducing agents on the low *p*H aggregate of BSA. Treatment of an aggregated sample in 0.10 *M* NaCl at the isoelectric *p*H with cysteine (4.4 moles per mole of BSA) for 2 days, or with potassium thioglycolate (1.0 mole per mole of BSA) for 1 day, caused no decrease in the extent of aggregation.

It was found that a reduction in aggregate content can be achieved by passing a solution containing the aggregate through an ion exchange column containing adsorbed thioglycolate. Columns were prepared using Rohm and Haas resins IR 120 and IRA 400, thoroughly washed with distilled water before use, and having the composition listed in Table VIII.

TABLE VIII

COMPOSITION OF GLYCOLATE COLUMNS

Layer	1 (top)	2	3	4 (mixed bed)		5
Vol., ml.	15	20	30	20	30	10
Form	NH_4^+	$HSCH_2COO^-$	CH_3COO^-	H^+	OH^-	H^+

The contact time of the protein solution with the thioglycolate section of the column, as estimated from the flow rate of the solution and the void vol-

(16) F. Pepe and S. J. Singer, THIS JOURNAL, **78**, 4583 (1956).

ume of the section, was found to be an important variable, as illustrated by the data in Fig. 2. It would appear that a contact time of several hours would be required to achieve complete conversion to the monomer. It was demonstrated by material balances that the apparent decrease in aggregate content was not the result of adsorption on the column. Thus in the experiment shown in Fig. 2,

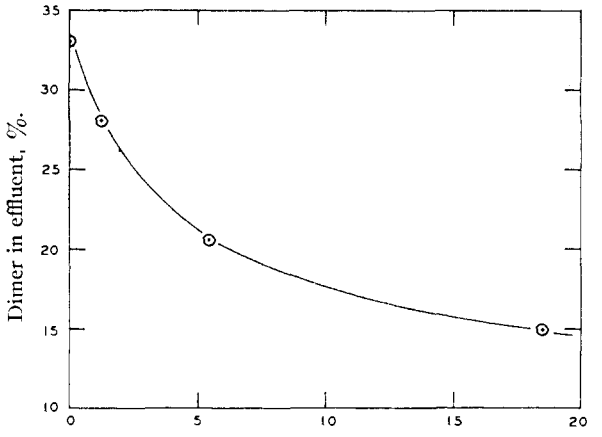


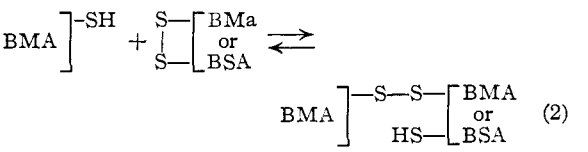
Fig. 2.—The conversion of the low *p*H aggregate of BSA to the monomer form on contact with a resin containing adsorbed thioglycolate.

96% of the protein was recovered, whereas had the indicated reduction of aggregate content been due to adsorption, the recovery would have been only 79%.

Discussion

The results which we have obtained clearly distinguish the low *p*H aggregation of BSA from the mercury dimerization of mercaptalbumin,[11] and from the oxidative disulfide dimerization of mercaptalbumin.[12] The salient differences include: (a) stability with respect to *p*H, the low *p*H dimer being unstable above *p*H 3.4, while the others are stable at neutral *p*H; (b) behavior toward excess Hg^{++}, which reverts the low *p*H dimer to monomer only very slowly, reverts the mercury dimer very rapidly,[11] and has no effect on the disulfide dimer; and (c) behavior toward cysteine, which has no effect on the low *p*H dimer, but reverts both the mercury and disulfide dimers to monomer.

The erratic behavior of the low *p*H aggregation reaction makes it appear likely that there is involved a catalytic mechanism which we have been unable to identify or control. Nevertheless, there is definite evidence that the sulfhydryl group of mercaptalbumin is involved, since: (a) a stoichiometric excess of Hg^{++} inhibits and slowly reverses the dimerization reaction; (b) the maximum extent of aggregation is greater with pure BMA than with a mixture of 57% BMA and 43% BSA; and (c) BSA with its sulfhydryl blocked does not aggregate. Furthermore, the monomerization of the dimer on a thioglycolate column suggests that the aggregation is the result of the formation of intermolecular disulfide bonds. Since the reaction takes place in the absence of any oxidizing agent other than the protein itself, it is proposed that a thiol–disulfide exchange is involved.



Thiol–disulfide exchanges have been observed or hypothesized in several systems. Ryle and Sanger[17] studied reactions of the type

$$R—S—S—R + R'—S—S—R' \rightleftharpoons 2R—S—S—R' \quad (3)$$

with cystine and various of its substituted derivatives. They found the reaction to be fairly rapid in 10 *N* HCl and very rapid in alkaline solutions, but to have a vanishingly low rate in weakly acid solutions. Since the alkaline reaction was strongly catalyzed by mercaptide ions, for example from cysteine, they concluded that it proceeds in the absence of added mercaptide ions by way of the formation of a small amount of such ion by the reaction

$$R—S—S—R + OH^- \rightleftharpoons R—S^- + R—SOH \quad (4)$$

The RS^- ion then attacks R'SSR' in a thiol–disulfide interchange. The reaction in strongly acid solution is inhibited by the addition of thiols, so that a different mechanism certainly applies in this case. Fava, Iliceto and Camera[18] employed S^{35} to follow the reaction between various mercaptans and the corresponding disulfides, and concluded from their kinetic data that the reaction is first order in RSSR and RS^-.

Huggins, Tapley and Jensen[19] studied the formation of gels by BSA and human serum albumin in concentrated solutions in the presence of urea, and presented convincing evidence that thiol–disulfide interchange is involved. Kauzmann and Douglas[20] have interpreted their data on the denaturation of BSA by urea in terms of the Huggins–Tapley–Jensen exchange reaction.

Rapid thiol–disulfide exchange reactions have been observed only in neutral or alkaline solutions, whereas the exchange we are proposing as an explanation for the aggregation of BSA occurs at *p*H values below 3.4. The non-occurrence of the reaction at higher *p*H values, in the absence of a denaturating agent, may be correlated with the numerous indications that BSA undergoes a reversible conformational change, which may be described as a swelling, at low *p*H. According to Tanford, *et al.*,[21] BSA exists in a compact form at *p*H values between 4.3 and 10.5. Just below *p*H 4.3 it changes to an expandable form which then undergoes a continuous expansion which increases with charge and decreases with ionic strength. Large changes in viscosity, sedimentation constant and other properties, resulting from this expansion, set in as the *p*H is dropped below about 3.5. Presumably the disulfide bond which is involved in the aggregation reaction is masked in some way until the

(17) A. P. Ryle and F. Sanger, *Biochem. J.*, **60**, 535 (1955).

(18) A. Fava, A. Iliceto and E. Camera, THIS JOURNAL, **79**, 833 (1957).

(19) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951).

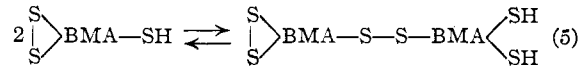
(20) W. Kauzmann and R. G. Douglas, Jr., *Arch. Biochem. Biophys.*, **65**, 106 (1956).

(21) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, THIS JOURNAL, **77**, 6421 (1955).

molecule has undergone a significant degree of swelling.

It may be pointed out that there are other reactions which take place in weakly acid solutions which may involve thiol–disulfide interchange. The sulfhydryl enzymes ficin[22] and papain[23] require activation by a reducing agent such as cysteine, and this activation can be produced at *p*H values as low as 4. Gorin, Dougherty and Tobolsky[24] have postulated that in general thiol–disulfide interchange is an intermediate step in the reduction of a disulfide by an excess of mercaptan. If this is true also in the activation of ficin and papain, these reactions serve as examples of exchange reactions proceeding rapidly in weakly acidic solution.

The slow reversal of the aggregation by raising the *p*H or by addition of $HgCl_2$ suggests that a monomer–dimer equilibrium exists, although it is evident that unknown factors exert an apparently erratic influence on either the rate of attainment of equilibrium or the extent of reaction at equilibrium, or in both. However on the assumptions that only the protein components are involved in the equilibrium state, we can try to distinguish between the following two possibilities: (1) BMA can react with itself or with BSA to give aggregates; or (2) BMA can react only with itself. The experimental data indicate that with pure BMA a maximum of 87% of dimer is formed at "equilibrium," while with ordinary BSA (57% BMA and 43% BSA), a maximum of 65% dimer is formed. For case 1, the figure for pure BMA leads to an equilibrium constant for the reaction (eq. 5) of $2K = 1.72 \times 10^5$ liters per mole, where K is the intrinsic constant for the reaction, assumed in what follows to be the same for



(5)

the disulfide group of both BMA and BSA. From this equilibrium constant we can calculate that the expected maximum yield of dimer from ordinary BSA is 70%. For case 2 a similar calculation leads to a maximum yield of only 47%. Thus this interpretation supports the view that the disulfide involved in the exchange can be supplied by either BSA or BMA.

Markus and Karush[25] have shown that a certain one of the 17 disulfide bonds in human serum albumin is particularly susceptible to reduction. Only this bond is reduced by mercaptoethylamine at *p*H 7 in the absence of detergent, whereas all the others undergo reduction when detergent is added. If bovine and human albumins behave similarly in this respect, it is an attractive hypothesis to assume that the thiol–disulfide exchange leading to aggregation at low *p*H involves the one sensitive disulfide bond in BSA.

The question arises as to the sources of the favorable free energy change accompanying the low *p*H aggregation reaction. In the mechanism proposed in equation 2, only an exchange of bonds occurs, and no new bonds are formed. Furthermore, there are factors which are adverse to the dimerization reaction. At low *p*H, BSA carries a large net positive charge, so that there is an electrostatic repulsion between BSA molecules which must be overcome in the formation of a dimer. In addition, one expects a considerable decrease in entropy in a protein dimerization reaction, primarily as the result of loss of translational and rotational entropy. One may speculate that any of a variety of additional factors may be involved, short-range dipole interactions, "hydrophobic" forces, etc., which have been proposed in other protein–protein interactions, but it is further possible in this case that when the intramolecular S–S bond is broken and replaced by an intermolecular bond, conformational changes may occur in the polypeptide chains near the original bond, which contribute to a favorable free energy change for the dimerization reaction.

(22) S. A. Bernhard and H. Gutfreund, *Biochem. J.*, **63**, 61 (1956).
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(24) G. Gorin, G. Dougherty and A. V. Tobolsky, THIS JOURNAL, **71**, 3551 (1949).
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NEW HAVEN, CONN.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, WEIZMANN INSTITUTE OF SCIENCE]

Poly-hydroxy-L-proline

BY JOSEPH KURTZ, GERALD D. FASMAN,[1] ARIEH BERGER AND EPHRAIM KATCHALSKI

RECEIVED JULY 31, 1957

O-Acetyl-hydroxy-L-proline (II) yielded on treatment with phosgene the intermediate N-carbonyl chloride III which was cyclized by means of silver oxide to O-acetyl-N-carboxyhydroxy-L-proline anhydride (IV). Poly-O-acetylhydroxy-L-proline (V) was derived from IV on polymerization in pyridine. Deacetylation of V with aqueous ammonia gave rise to polyhydroxy-L-proline (VI). Osmotic and sedimentation measurements in aqueous solution gave average molecular weights of 10,600 and 10,700, respectively, for the sample of VI synthesized. O-*p*-Tolylsulfonylhydroxy-L-proline (X) was synthesized and converted into the corresponding N-carboxyanhydride XI, by means of phosgene and silver oxide. Poly-O-*p*-tolylsulfonylhydroxy-L-proline (XII) was obtained by polymerization of XI in pyridine. V showed mutarotation in formic acid. The specific optical rotation changed within several hours from +25° to −175°. The form with $[\alpha]^{25}D$ −175° could be reversed to the form with $[\alpha]^{25}D$ +25° by means of dimethylformamide. Mutarotation in acetic acid was observed also with XII. In this case reversal could be effected by means of pyridine.

In a previous publication[2] the synthesis of poly-L-proline was reported. This compound proved to be a useful high molecular weight model in the study of collagen and gelatin.[3] As collagen also

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(2) A. Berger, J. Kurtz and E. Katchalski, THIS JOURNAL, **76**, 5552 (1954).
(3) P. M. Cowan and S. McGavin, *Nature*, **176**, 501 (1955); P. M. Cowan, S. McGavin and A. C. T. North, *ibid.*, **176**, 1062 (1955).