same sign (though, of course, the protein bears both plus and minus charges and doubtless has regions of net *positive* charge) and also because ring substitutions[29,30] manifestly influence the strength of binding. It is also well known that divalent cations, particularly Mg^{++}, influence the extent of binding.

In the previous work of Blum,[11] which has been confirmed and extended in the course of the present investigation, the electrostatic explanation has received at least some indirect support, in that effects rather similar to those brought about by ATP can be evoked by imposing fairly high (>10) *p*H's[31]: Although the number of proton-binding sites certainly exceeds the number of ATP binding sites, the effects brought about by high *p*H must unequivocally result from the imposition of an electrostatic stress onto the protein particle. At the same time it must be conceded that the electrostatic explanation of the ATP effect cannot at this time be put quantitatively. Measurements of the number of ATP binding sites, albeit dissonant and not on the firmest theoretical basis,[32] suggest that saturation with ATP would not sufficiently alter the *net* charge to bring about the considerable structural changes observed, so that the electrostatic explanation must also be combined with some assumptions about strategic location of the ATP binding sites.

A third question on which this work bears has to do with the general composition of the myosin B system. The polydispersity of the system, and more specifically its distribution into a minimum of three size classes, has been demonstrated, and it also has been shown that polymerization (or perhaps in some instances copolymerization) reactions connect these various classes. These reactions have been shown to be easily displaceable by concentration, *p*H and temperature changes; this property probably rules out polymerization through covalent bonding. Thus the myosin B system appears to resemble comparable aggregating protein systems, except that the particles of the myosin B system seem specialized to adsorb polyphosphates such as ATP, thus providing a special means of displacing the polymerization reactions.

(29) J. J. Blum, *Arch. Biochem. and Biophys.*, **55**, 486 (1955).

(30) E. T. Friess and M. F. Morales, *ibid.*, **56**, 326 (1955).

(31) The reproducibility achieved to date by adding KOH to myosin B solution (to *p*H 11) is inferior to that attained with ATP addition. However, in several Zimm-type light-scattering experiments, the similarity with the ATP result is striking. For example, in one such case, (with 5-hr. extracted myosin B) the weight average molecular weight of the particles remained almost constant ($\overline{M}_w$ went from 22.2×10^6 to 20.0×10^6), while the average radius of gyration, $\bar{r}_g$, rose sharply (from 3310 to 4270 Å.). It is interesting that the stress imposed in such experiments cannot be borne indefinitely. Thus, in this case, after 45 minutes at high *p*H, $\overline{M}_w$ had fallen to 17.5×10^6 and $\bar{r}_g$ to 4030 Å.

(32) M. F. Morales, J. Botts, J. J. Blum and T. L. Hill, *Physiol. Revs.*, **35**, 475 (1955).

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Molecular Weight Studies on Human Serum Albumin after Reduction and Alkylation of Disulfide Bonds[1]

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The disulfide groups of human serum albumin were reduced by sodium thioglycolate in the presence of urea or sodium dodecyl sulfate to render all the disulfide bonds available for reaction. After reduction, an excess of iodoacetamide was added to block the protein sulfhydryl groups and prevent their subsequent reoxidation. Viscosity, light scattering and ultracentrifuge studies were performed on the reduced protein and on an unreduced control. Upon correcting for bound detergent, the molecular weight of the protein appeared unchanged. No amino acids nor small peptides were found in the dialysates, and the protein solution appeared homogeneous in the ultracentrifuge. The appearance of carboxy-S-methylcysteine and the disappearance of cystine in the hydrolysates of the reduced protein was confirmed by paper chromatography. The results suggest that the human serum albumin molecule is a continuous peptide chain internally crosslinked by disulfide bonds.

I. Introduction

Amino acid end-group analyses of human serum albumin have shown the presence of only one terminal amino group per molecule[3,4] and studies with carboxypeptidase have indicated that only one terminal carboxyl group is present.[5] While this evidence might suggest that the albumin molecule is composed of a single polypeptide chain, it is not conclusive as cyclic peptides, interlinked by disulfide bonds, could be present in the native protein.

The existence of cyclic peptides, at least in bovine serum albumin, would appear to have been demonstrated by the studies of Reichmann and Colvin.[6,7] These workers have shown that oxidation of the disulfide bonds of bovine serum albumin with performic acid resulted in the appearance of several lower molecular weight species as determined by ultracentrifugal analyses and light scattering measurements.

It seemed of interest to determine whether similar results would be obtained on rupture of the disulfide bonds by reduction rather than by oxidation and the present study was therefore undertaken to determine the molecular weight of thioglycolate-reduced human serum albumin. Disulfide bonds can be reduced by thiol compounds under some-

(1) This work has been supported by funds of Harvard University, by grants from the National Institutes of Health, and by contributions from industry.

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(3) E. O. P. Thompson, *J. Biol. Chem.*, **208**, 565 (1954).

(4) H. Brown, *Science*, **121**, 106 (1955).

(5) W. F. White, J. Shields and K. C. Robbins, THIS JOURNAL, **77**, 1267 (1955).

(6) M. E. Reichmann and R. Colvin, *Can. J. Chem.*, **33**, 163 (1955).

(7) M. E. Reichmann and R. Colvin, *ibid.*, **34**, 160 (1956).

what milder conditions than those employed in performic oxidations. Moreover, reduction by thiol compounds has the added advantage that in proteins it is specific for cystine residues.

The reduction of the disulfide bonds of human serum albumin by thiol compounds has been studied in some detail.[8,9] Using β-mercaptoethylamine as a reducing agent, Markus and Karush[9] found that at *p*H 7.4 less than one disulfide bond per molecule of albumin was split, although on the addition of a detergent (sodium decyl sulfate) all the protein disulfide bonds could be reduced.

Furthermore, despite the fact that the reaction

$$\text{cystine} + \text{thioglycolate} \rightleftarrows \text{cysteine} + \text{dithioglycolate}$$

has an equilibrium constant of the order of one,[10,11] Katchalski and Benjamin[8] were unable to reduce any of the disulfide bonds of human serum albumin between *p*H 5.5 and 7.0 even in the presence of a hundred-fold excess of thioglycolate. Preliminary experiments in this Laboratory, however, showed that addition of urea rendered the disulfide bonds available for reduction and that complete reduction of the groups could be obtained when the urea concentration was raised to 8 *M*.

As complete reduction of the cystine bridges was desired in the studies described herein, all reductions were carried out either in the presence of 8 *M* urea or sodium dodecyl sulfate (SDS). In most of the following experiments sodium dodecyl sulfate was used rather than urea since the presence of the necessary high concentrations of urea would have interfered with the detection of any small peptides or amino acids that were split off following reduction.

The reactions of sodium dodecyl sulfate[12] and sodium decyl sulfate[13] with bovine and human serum albumin have been studied previously and appropriate detergent/protein ratios were chosen from the results of these studies. Re-oxidation of the protein sulfhydryl groups was prevented by the addition of excess iodoacetamide.

II. Materials and Methods

Preparation and Characterization of Human Serum Albumin.—The dried powder used as starting material in these experiments had been prepared by fractionation with alcohol and recrystallized three times.[14] An 8% solution of the powder was passed through a mixed-bed deionizing resin[15] and the resultant solution kept frozen at −5° when not in use. Electrophoretic analysis at *p*H 8.6 in Veronal buffer showed the solution to contain 97% albumin and 3% α-globulin. Ultracentrifugal analysis revealed 95% of one component, $S^0_{20,w} = 4.46$, and 5% of a faster component, $S^0_{20,w} = 6.5$. The molecular weight used in all calculations was 67,000. Titration with methyl mercury nitrate in the presence of guanidine bromide indicated that the albumin contained 0.3 sulfhydryl groups per molecule. All *p*H measurements were made at 25° on a Cambridge *p*H meter. The $E^{1\%}_{1cm.}$ at 280 mμ as determined from dry weight measurements was 5.31, in good agreement with the published figure of 5.30.[14] All measurements of optical density were made on a Beckman DU Spectrophotometer. The thioglycolic acid was distilled under vacuum at 125°, stored in a dark bottle at +1° and standardized with methyl mercury nitrate. Iodoacetamide was prepared by reacting chloroacetamide with sodium iodide.[16] It was recrystallized twice before use and stored in a dark bottle at +1°. The sodium dodecyl sulfate employed was "Duponol PC" (kindly supplied by E. I. du Pont de Nemours and Co.) referred to hereafter as SDS. It contained a small amount of near homologs.

The ultracentrifugal analyses were carried out in a Pickels–Bauer type air-driven ultracentrifuge, the light scattering measurements on a modified Debye instrument[17] and the electrophoretic analyses on a Tiselius type electrophoresis apparatus. Refractive index was measured on a Bausch and Lomb Dipping Refractometer.

Reduction of Albumin.—The SDS was added to the salt-free albumin solution, the *p*H adjusted to 8.2 with 2% sodium carbonate and the solution allowed to stand at room temperature for an hour. Sodium thioglycolate solution (*p*H 8.2) was then added, the *p*H adjusted to 8.2–8.5 and the flask immediately evacuated and filled with nitrogen. The concentrations of the various reagents[18] are shown in Table I.

TABLE I

REAGENTS FOR THE REDUCTION OF HUMAN SERUM ALBUMIN

(Final concentrations in the reaction mixture)

	Albumin	Thioglycolic acid	SDS
Concn. (g./100 ml.)	1.75	3.2	1.5
Molarity	2.6×10^{-4}	0.35	5.2×10^{-2}
Molar ratio $\frac{\text{reagent}}{\text{albumin}}$	.....	1350	200

The evacuation and addition of nitrogen was repeated twice and the mixture left in the dark for 20 hr. at room temperature.

Reaction of Reduced Albumin with Iodoacetamide.—A 1.25-fold excess of 0.5 *M* iodoacetamide was placed in a flask and the flask evacuated and filled with nitrogen. A stream of nitrogen was played on the surface of the solution containing the protein and thioglycolate, and the iodoacetamide solution added with continual stirring. 2 *N* sodium hydroxide was added concurrently to maintain the *p*H above 6. The *p*H was continually checked with indicator paper until it reached 8.0–8.5 and did not change on allowing the solution to stand for ten minutes. The flask was then evacuated, filled with nitrogen and left in the dark at room temperature for 5 hr. At the end of this time the *p*H was checked to make sure it had not become more acid and the completeness of the reaction determined by withdrawing a 0.5-ml. sample and testing for free sulfhydryl groups with nitroprusside. A negative test was always obtained. The mixture was dialyzed five times against 100 volumes of $\Gamma/2 = 0.05$ phosphate buffer, *p*H 7.45, for 24 hr. at 1°. This preparation will be referred to throughout this paper as reduced-SDS–albumin.

Control experiments were conducted in which the above procedure was followed except that thioglycolate was omitted from the mixture (referred to hereafter as SDS–albumin).

The albumin was also reduced in the presence of 8 *M* urea instead of SDS. The reduction was otherwise carried out in exactly the same manner. Control experiments in which the reducing agent was absent were also performed.

Estimation of SDS.—A modification of the method of Lundgren[19] was used to dissociate the protein–SDS complex. Five ml. of reduced-SDS–albumin and SDS–albumin were treated at room temperature with 2 ml. of 1 *N* NaCl, 10.5 ml. of acetone and a few drops of *p*H 3.9 acetate buffer to bring the *p*H to 5.0–5.3. This precipitated the protein while the SDS remained in solution. The suspension was centrifuged and the precipitate washed with 5 ml. of water,

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

(9) G. Markus and F. Karush, *ibid.*, **79**, 134 (1957).

(10) T. Bersin and J. Steudel, *Ber.*, **71B**, 1015 (1938).

(11) I. M. Kolthoff, W. Stricks and R. C. Kapoor, THIS JOURNAL, **77**, 4733 (1955).

(12) B. S. Harrap and J. H. Schulman, *Disc. Faraday Soc.*, No. 13, 197 (1953).

(13) G. Markus and F. Karush, THIS JOURNAL, **79**, 3264 (1957).

(14) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, *ibid.*, **69**, 1753 (1947).

(15) H. M. Dintzis, Dissertation for Ph.D. Thesis, Harvard University, 1953.

(16) M. L. Anson, *J. Gen. Physiol.*, **23**, 256 (1939–1940).

(17) W. B. Dandliker, THIS JOURNAL, **72**, 5110 (1950).

(18) If much higher concentrations of reagents were used, the solution formed a gel on the addition of thioglycolate.

(19) H. P. Lundgren, *Textile Research J.*, **15**, 335 (1945).

2 ml. of 1 *N* NaCl and 10.5 ml. of acetone. The suspension was recentrifuged and the two supernatants combined. The resultant solution was evaporated to dryness at 60° and the residue dissolved in a standard volume of water. Control experiments in which known amounts of SDS were added to albumin showed that the SDS could be recovered quantitatively (within an experimental error of about 5%) by this procedure. The SDS concentrations were measured by the method of Karusch and Sonenberg[20] as modified by Pallansch and Briggs.[21]

Estimation of the Number of Disulfide Bonds Broken on Reduction. 1. **Chromatography of the Protein Hydrolysate.**—The SDS–albumin solutions were treated with acetone as described in the previous paragraph. The protein precipitates were dissolved in 2.5 ml. of concentrated HCl and 2.5 ml. of 85% formic acid. The solutions were gently refluxed under nitrogen for 40 hr. after which time they were evaporated to dryness and dissolved in water to a final volume of 5 ml. Standard amino acid mixtures and a native albumin hydrolysate were run in parallel as controls. Cysteine hydrochloride was reacted with monochloroacetic acid to give carboxy-S-methylcysteine as described by Michaelis and Schubert[22] and aliquots of this were also run as controls.

Chromatographic analyses of the amino acids in the hydrolysates were perfomed in ammoniacal phenol–water and isopropyl alcohol–water[23] systems on Whatman No. 1 and No. 4 papers. The presence of sulfur-containing amino acids was demonstrated by the platinic iodide test.[24] All samples which were run in the phenol solvent were treated with 30% H_2O_2 to convert all the cystine to cysteic acid, since in this solvent system there is a partial spontaneous conversion of cystine to cysteic acid.

2. **Estimation of Amide Ammonia before and after Reduction.**—As only cysteine residues appeared to be attacked by iodoacetamide, any increase in the amide ammonia after reduction and alkylation was a measure of the number of disulfide bonds which had been reduced and converted to the thioether.

Various methods for hydrolysis of the amide groups of albumin were investigated, but the only technique giving consistently reproducible results which agreed with the figures for amide nitrogen in the literature[25] was a modification of the method reported by Rees.[26]

Four-ml. samples of reduced-SDS–albumin, SDS–albumin and native albumin were lyophilized overnight. Twenty ml. of concentrated HCl was added to each and the solutions allowed to stand in a 37° oven for 220 hr. At the end of this time the samples were neutralized with 19 *N* NaOH. The solutions were then transferred to a Van Slyke–Cullen urea apparatus, 50 ml. of saturated potassium carbonate added, the ammonia thus liberated collected in 0.01 *N* HCl and the excess HCl titrated with 0.01 *N* NaOH.

Oxidation of Human and Bovine Serum Albumin with Performic Acid.—Both human and bovine serum albumin were treated with performic acid to oxidize the cystinyl residues exactly as described by Reichmann and Colvin.[6,7] The products were then dialyzed extensively against $\Gamma/2$ = 0.01 NaCl, $\Gamma/2$ = 0.05 phosphate buffer, *p*H 7.45.

III. Results

Composition of SDS–Albumin Complexes.—The amount of SDS present in the dialyzed SDS–albumin complexes is shown in Table II.

It was noticed that on standing for several weeks lower values for the amount of SDS were obtained. At low temperatures the loss of SDS was minimized but was still observable over longer periods of time. The loss in SDS was paralleled by an increase in turbidity, and precipitation of the protein occurred on further standing. To avoid this, all analyses were performed within two weeks of the preparation of the reduced albumin. This behavior of SDS on standing has been observed by other workers[21] and is presumably due to hydrolysis.

TABLE II

AMOUNT OF SDS BOUND BY ALBUMIN (MOLES/MOLE)[27]

	Reduced	Control
Run I	94	67
Run II	92	64
Run III	93	63

Removal of SDS from Reduced Albumin.—Removal of SDS from the protein by treatment with acetone as previously described gave a protein precipitate which appeared denatured being no longer soluble at *p*H 7.5. Alternatively, the SDS–albumin complex was treated with barium chloride to remove the SDS from the protein as described by Putnam and Neurath.[28] While an SDS-free, soluble protein could readily be obtained from the unreduced SDS–albumin, the solution of a reduced albumin became very opalescent after precipitation of the detergent as the barium salt. On centrifugation of this opalescent solution at 27,000 *g* a glass was obtained, the supernatant being essentially free of protein.[29] Raising the ionic strength of the reduced-SDS–albumin to 0.15 caused the solution to become slightly opaque, and analysis in the ultracentrifuge showed the appearance of much rapidly-sedimenting material. It has been shown that the amount of SDS bound by albumin is dependent on the ionic strength of the solution,[12] and it seems probable that the higher salt concentration reduced the amount of bound SDS to a level at which the protein was no longer soluble at neutral *p*H values. All physical measurements on the SDS–albumins were therefore performed on samples which had been extensively dialyzed against $\Gamma/2$ 0.05 phosphate buffer, *p*H 7.45.

To make sure that the SDS was in fact protein-bound and did not exist, at least to any great extent, as undialyzable SDS micelles, electrophoretic analyses were performed on SDS, reduced-SDS–albumin, and reduced-SDS–albumin to which varying amounts of SDS had been added. The analyses were run in $\Gamma/2$ = 0.05 phosphate buffer, *p*H 7.45 at 20°. The reduced-SDS–albumin moved as a single component with no noticeable boundary spreading and with a much slower mobility than that of the SDS micelle. On adding small amounts of SDS to the reduced-SDS–albumin, a single peak was still obtained, the mobility increasing with increasing SDS concentration. On the addition of large amounts of SDS to the albumin, a double peak appeared to be present but the mobility of the reduced-SDS–albumin was, by this time, so close to the mobility of the SDS micelle that it was difficult to obtain good separation. From these results it seemed unlikely that SDS micelles were present to any appreciable amount

(20) F. Karush and M. Sonenberg, *Anal. Chem.*, **22**, 175 (1950).

(21) M. J. Pallansch and D. R. Briggs, THIS JOURNAL, **76**, 1396 (1954).

(22) L. Michaelis and M. P. Schubert, *J. Biol. Chem.*, **106**, 340 (1934).

(23) R. H. McMenamy, C. C. Lund and J. L. Oncley, *J. Clin. Invest.*, **36**, 1672 (1957).

(24) G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).

(25) E. Brand, J. T. Edsall, *Ann. Rev. Biochem.*, **16**, 223 (1947).

(26) M. W. Rees, *Biochem. J.*, **40**, 632 (1946).

(27) The final dialysate contained less than 10^{-3} mole SDS/ml.

(28) F. W. Putnam and H. Neurath, THIS JOURNAL, **66**, 692 (1944).

(29) The samples reduced in the presence of urea showed a similar solubility behavior on the removal of the urea by dialysis against $\Gamma/2$ = 0.05 phosphate buffer, *p*H 7.5.

in the reduced-SDS–albumin solutions. The SDS could therefore be considered protein-bound.

Estimation of Sulfhydryl Groups Formed on Reduction. 1. Chromatographic Estimation of Cystine and Carboxy-S-methyl-cysteine in the Protein Hydrolysates.—Control experiments with synthetic carboxy-S-methylcysteine showed that this compound could not be satisfactorily separated from other amino acids in either of the solvent systems which were used. It could however readily be separated from the other sulfur-containing amino acids and identified by spraying the papers with platinic iodide. Carboxy-S-methylcysteine was found to be present in approximately theoretical concentration in the hydrolysates of the reduced protein.

The almost complete disappearance of cystine from the hydrolysates of the reduced protein could be demonstrated in the ammoniacal phenol–water system. Quantitative determinations, performed by comparison with known amounts of cystine, revealed that less than 2% of the original amount of cystine was still present in the reduced protein (less than 0.3 mole per mole of albumin).[30] The amount of cystine recovered from the unreduced albumin on the other hand agreed within 5% of the published values.

2. Estimation of Amide Ammonia before and after Reduction.—Analyses of SDS–albumin and native albumin showed the protein to contain 41–42 amide groups per mole, results which agree well with the published figures[25] (42 moles/mole). Samples from three different reductions of albumin contained 75, 79 and 79 moles of amide ammonia per mole of protein. The difference between the control and reduced samples (33–37 moles per mole) compares favorably with the 35 half-cystines present in the albumin molecule.[25]

Analysis for Amino Acids or Small Peptides Liberated on Reduction.—Ten ml. of the undialyzed, alkylated protein was dialyzed overnight against 20 ml. $\Gamma/2 = 0.05$ phosphate buffer, *p*H 7.45 at +1°. The dialysate was equilibrated with Dowex 50 on the hydrogen cycle (15 g. wet resin) for 45 minutes, the resin washed with distilled water and any bound material eluted with 5 *N* ammonia. The ammonia solution was evaporated to dryness and the residue dissolved in 2 ml. of water. The solution was ninhydrin and biuret negative.

In another experiment, 20 ml. of the dialysate was lyophilized, refluxed with 6 *N* HCl for 36 hr., evaporated to dryness and the residue dissolved in 20 ml. of water. The solution was treated with Dowex 50 as described above and subjected to chromatographic analysis. No amino acids could be detected after chromatographic analysis and the solution was ninhydrin negative.

Molecular Weight Determinations. 1. Viscosity and Sedimentation Molecular Weights. a. Ultracentrifugal Analysis of Reduced-SDS-Albumin and SDS–Albumin.—The ultracentrifuge runs were performed in $\Gamma/2 = 0.05$ phosphate buffer, *p*H 7.45 at various protein concentrations. Both SDS proteins were approximately 95% homogeneous with about 5% of a faster-sedimenting material. A typical picture is shown in Fig. 1.

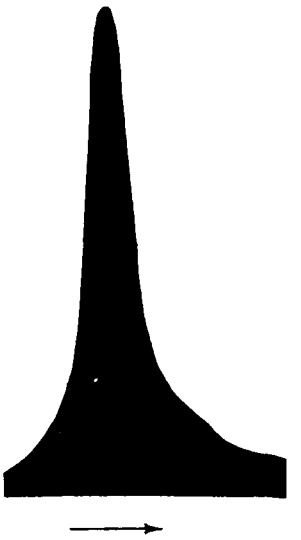

Fig. 1.—Ultracentrifugal diagram of reduced-SDS-albumin at 120 min.

The $S°_{20,w}$ for the reduced-SDS–albumin extrapolated to values ranging from 3.8 to 4.0 at zero concentration as shown in Fig. 2 while the control SDS–albumin gave a value of 4.24.

Although there was no sign of slower-sedimenting material even at the lowest protein concentrations, it was thought that the SDS might be acting as an interchain bonding agent and mask any dissociation that had occurred after rupture of the disulfide bonds. No such aggregation should occur in the albumin solutions which had been reduced in the presence of 8 *M* urea. On ultracentrifugal analysis in 8 *M* urea, *p*H 7.5, $\Gamma/2 = 0.05$, these solutions showed a single component with a sedimentation constant of the same order of magnitude as the unreduced control. While precise ultracentrifugal analyses are difficult in 8 *M* urea due to the curvature of the base line, it should have been possible to detect any slower sedimenting material had it been present in concentrations greater than 5%.

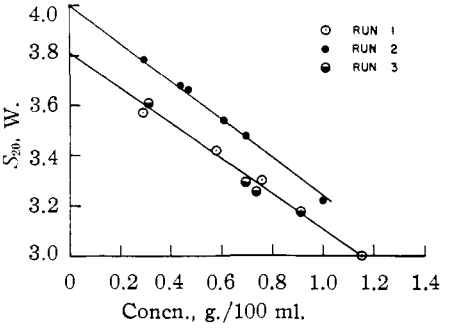

Fig. 2.—Ultracentrifugal analyses of reduced human serum albumin.

b. Viscosity Measurements.—The viscosities of the various solutions were determined in $\Gamma/2 = 0.05$ phosphate buffer, *p*H 7.45. Four-ml. samples were run in Fenske–Ostwald viscometers (water flow time *ca.* 260 sec.) in a constant-temperature bath at 25 ± 0.01°. Density measurements were performed in pycnometers at the same temperature and protein concentrations were obtained from dry weight determinations.[31] From these measurements, the intrinsic viscosity was obtained by extrapolation to zero concentration as shown in Fig. 3.

The plot $\ln \eta/\eta_0$ against $1/c \ln \eta/\eta_0$ was used[32] instead of the more conventional plot of $1/c \ln$

(30) Similar results were obtained with albumin reduced in the presence of 8 *M* urea.

(31) The dry weight of the buffer solution was determined and subtracted from that obtained for the protein solution.

(32) J. Foster, *J. Phys. Colloid Chem.*, **53**, 175 (1949).

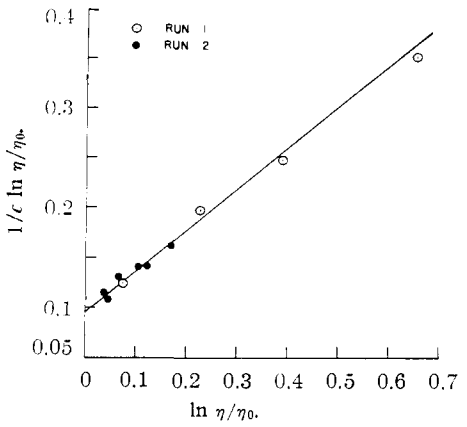

Fig. 3.—Intrinsic viscosity of reduced human serum albumin.

η/η_0 against c as the curve given by the latter plot was difficult to extrapolate with certainty. The shape of this latter curve seemed to indicate that considerable intermolecular interaction was occurring at higher protein concentrations.

The value thus obtained for the intrinsic viscosity, $[\eta]$ of the reduced albumin was 0.095. A few measurements were made on the control SDS–albumin giving an intrinsic viscosity of 0.056. The relative viscosity of the control samples was relatively independent of protein concentration and resembled much more closely the values obtained for native albumin.

The partial specific volumes, $\bar{v}$, of the SDS–albumin complexes were calculated from the published values for albumin and SDS. The partial specific volume of albumin was taken as 0.733[33] while that of the SDS was taken as 0.87.[34] In two instances the partial specific volume of the reduced-SDS–albumin was determined from density measurements and gave results which agreed within 3% of the calculated value.

From the above information ν, the Einstein viscosity increment, was calculated from

$$\nu = \frac{[\eta]\ 100}{\bar{v}}$$

Using this value, the axial ratio, a/b, was obtained from tables for prolate ellipsoids[35] and the contour maps of Oncley.[36,37] The proteins were assumed to have 30% hydration.[38]

Hence, f/f_0, the frictional ratio was determined.[36] The molecular weight, M, was then calculated from the equation[36]

$$M = 2.45 \times 10^{22} \bar{v}^{1/2} \left[\frac{f/f_0 \times S^0_{20,w}}{1 - 0.9982\bar{v}}\right]^{3/2}$$

(33) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

(34) G. L. Miller and K. J. I. Anderson, *J. Biol. Chem.*, **144**, 475 (1942).

(35) J. W. Mehl, J. L. Oncley and R. Simha, *Science*, **92**, 132 (1940).

(36) J. L. Oncley, *Ann. N. Y. Acad Sci.*, Vol. XLI, 121 (1941).

(37) The contour maps of Oncley were computed for molecules with a $(1/\bar{v}\rho)$ value of 1.34, an appropriate value for most proteins. The detergent–Albumin complexes had lower values of $1/\bar{v}\rho$ and corrections in the degree of hydration were made to compensate for this lower value before the contour maps were used to obtain f/f_0 values.

(38) No experiments were conducted to determine the degree of hydration of the SDS-albumins. If no hydration was assumed, the molecular weight was lowered by about 4000 and a variation of this magnitude would not affect the conclusions which were reached as a result of the molecular weight determinations.

TABLE III

SEDIMENTATION AND VISCOSITY DATA FOR REDUCED-SDS–ALBUMIN AND SDS–ALBUMIN

	SDS-albumin	Reduced SDS-albumin
$\bar{v}$	0.760	0.770
$[\eta]$	0.056	0.095
ν	7.37	12.34
f/f_0	1.37	1.54
$S^0_{20,w}$	4.24	3.82
		4.01
Mol. wt. of SDS–albumin	80,000	87,000
		94,000
Moles SDS/mole albumin	64	94
Mol. wt. albumin	63,000	62,000
		69,000

The values obtained are summarized in Table III.

2. Light Scattering Molecular Weight.—The protein solutions were clarified by centrifugation in a high speed head of an International Centrifuge at 20,000 g for 3 hr. at 3°. The top solutions were carefully pipetted off and transferred to clean light scattering cells. The scattering at 90° was measured at various protein concentrations. The intensity of the 90° scattering for the buffer solutions was never more than 1% of the scattering for a 1% protein solution. The intensity of the transmitted beam was determined at 0°. All measurements were calibrated on an absolute scale by reference to a Ludox standard.[39] The results of the light scattering studies are shown in Fig. 4.[40]

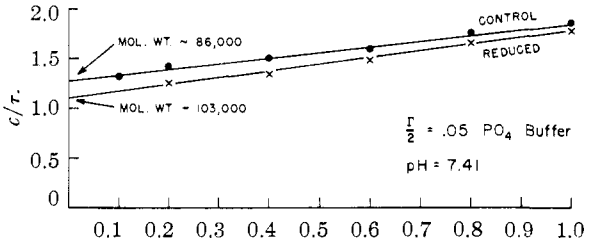

Fig. 4.—Light scattering analysis of reduced and control human serum albumin.

The molecular weight of the control albumin sample, after correction for bound detergent, was thus 69,000, while that of the reduced protein was 78,000.

Sedimentation Rates of Oxidized Human and Bovine Serum Albumin.—The proteins were dialyzed against 0.08 M borate, 0.2 M NaCl, pH 7.4 (as described by Reichmann and Colvin[6,7]) before ultracentrifugal analysis.

The performate oxidized human serum albumin gave a sedimentation pattern (using schlieren optics) identical with that of the bovine. Both protein solutions showed a very broad peak, obviously inhomogeneous, with a mean $S_{20,w}$ of about 1.5. This value agrees well with that reported by Reichmann and Colvin ($S^0_{20,w} = 1.49 \pm 0.05$) for oxidized bovine albumin.

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IV. Discussion

The importance of the disulfide crosslinks in maintaining the native three-dimensional structure of human serum albumin and in reversing the action of denaturing agents after the latter's removal was very apparent in these studies. For example, although the initial protein/SDS ratio was the same for both the reduced and control albumin, the dialyzed, reduced protein bound one and a half times as much SDS as did its unreduced counterpart. The detailed studies of Markus and Karush[5,10] on the viscosity and optical rotatory changes that occur after the addition of β-mercaptoethylamine to albumin–detergent solutions have indicated that large changes occur in the three-dimensional protein structure following rupture of the disulfide bonds and the present studies have shown a similar increase in viscosity following reduction. Furthermore, the behavior of the reduced protein was markedly different from that of the control after removal of the denaturing agent, whether urea or SDS. Removal of these reagents from the control albumin solution yielded a protein closely resembling the native molecule, whereas the reduced protein was completely insoluble except in strong acid or base after removal of the denaturing agent.

Since the homogeneity of the SDS–albumin and reduced-SDS–albumin solutions, as determined by ultracentrifugal and electrophoretic analyses, was comparable to that of the native protein, the amount of SDS bound by the protein could not have varied significantly from molecule to molecule. By neither method was a series of complexes observed as described by Neurath and Putnam[41] in studies on horse serum albumin. Nor was there any evidence for the existence of free SDS micelles. All the detergent could therefore be considered as protein-bound. Working with bovine serum albumin at similar salt concentrations, Cockbain[42] found that micelle formation was not evident until the detergent/protein ratio reached 220, a much higher ratio than was present in the dialyzed protein solution.

It was therefore possible to treat the SDS–albumin complex as a single entity and to determine the molecular weight of the aggregate in a reliable manner. The molecular weight of the reduced protein (after correction for bound detergent) agreed well with the published values for the native protein thus excluding the presence of appreciable amounts of lower molecular weight species in these preparations.

Furthermore, since no amino acids or small peptides could be detected in the protein dialysates after reduction, it would appear that the entire molecule was composed of a single polypeptide chain.[43] The disulfide bonds must therefore all be present as intra-chain crosslinks.

These results are in disagreement with those obtained by Reichmann and Colvin[6,7] on performate-oxidized bovine serum albumin. As previously mentioned, these workers found several lower molecular weight species present in their oxidized preparations. To ensure that these contradictory results were not due to the different species of albumin used in the two studies, both human and bovine serum albumin were oxidized with performic acid in exactly the manner employed by Reichmann and Colvin. The preparations looked identical on ultracentrifugal analysis, both showing several lower molecular weight species with the same mean sedimentation rate as described by the above authors. The disagreement would therefore appear to be due to the method employed in the rupture of the disulfide bonds.

That the higher molecular weight obtained in the present study was due to incomplete reduction of the cystine residues appears very unlikely. On repeated chromatographic analyses of the reduced protein hydrolysates, only a trace of cystine could be detected when the hydrolysate was applied to the paper at high concentrations. Furthermore, since hydrolysates of the control samples showed the cystine to be present in theoretical concentrations, no destruction of cystine could have occurred during hydrolysis. At most, the reduced preparations contained about a third of a mole of cystine per mole of protein.

There is a possibility that SDS could replace cystine as an intra-chain crosslinking agent and mask any dissociation that had occurred. Any such aggregation however should not be present in the urea-treated protein solution where again a single component was obtained on ultracentrifugal analysis.

There was some indication that changes other than oxidation of the disulfide bonds had occurred in the studies with performic acid. The ultraviolet absorption spectra of both oxidized bovine and oxidized human serum albumin were very different from those of the native proteins. The E_{max} had shifted to a lower wave length and increased greatly in magnitude. This alteration is presumably due to oxidation of, or substitution in, the tyrosyl residues,[44] a change which in itself however need not result in peptide bond rupture. Tryptophan is rapidly destroyed by performic acid[45] and the presence of any protein tryptophanyl residues might well result in splitting of the peptide chain on oxidation of the protein. Human serum albumin, however, contains only one tryptophanyl residue per mole while bovine serum albumin has been reported as containing two.[46] Oxidation of these residues with concomitant peptide bond rupture would not appear to be sufficient to account for the three or four molecular species obtained on oxidation in the case of the human serum albumin.[47] Furthermore, performic oxidation of hemoglobin, a protein containing five or six tryptophanyl

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residues, has not shown the presence of lower molecular weight species which could be attributed to peptide bond rupture at the tryptophanyl residues.[48]

In conclusion, the studies with thioglycolate described herein have shown the reduced human serum albumin to be a homogeneous protein of the same molecular weight as the native molecule. The disulfide bonds are therefore all present as intrachain crosslinks. The reason for the difference between the oxidized and reduced preparations is as yet unclear.

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Near Infrared Spectra of Compounds with Two Peptide Bonds and the Configuration of a Polypeptide Chain. VII. On the Extended Forms of Polypeptide Chains

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Infrared absorptions of the five acetylamino acid N-methylamides (where amino acids are glycine, alanine, valine, norleucine and proline) in the 3 μ region have been observed with a grating spectrometer. The results are interpreted in terms of the molecular configurations of these compounds (the folded and the extended forms). Our previous conclusion for the folded form in these solutions has been confirmed. As to the extended form more accurate measurement has shown that this form of acetylglycine N-methylamide is different from that of acetylalanine (or valine or norleucine) N-methylamide. Taking this difference into account, the molecular configurations of polyglycine and other polypeptides consisting of L-amino acid residues have been discussed.

Introduction

The near infrared spectra of acetylamino acid N-methylamides, $CH_3CONHCHRCONHCH_3$, have been measured in carbon tetrachloride and chloroform solutions.[1-8] Based on these results, the molecular structure of these substances has been discussed in relation to the configuration of a polypeptide chain.

Recently spectrometers with higher resolving power and higher accuracy become available and many of the spectra have now been re-examined. A number of new facts have been observed, most of which have confirmed our former conclusions, and none of which have led to any essential alteration of them. However, some of the new results have enabled us to discuss in more detail the molecular structure of acetylamino acid N-methylamides and hence also of polypeptides.

In this paper, the results of the re-examinations of the near infrared spectra of five acetylamino acid N-methylamides, where, the amino acids are glycine, DL-alanine, L-valine, DL-norleucine, and L-proline in carbon tetrachloride will be given. The solutions are so dilute that intermolecular hydrogen bonding is negligible.

Re-examinations of the spectra of more concentrated solutions will be given later.

Experimental

The samples used are acetylglycine N-methylamide, acetyl-DL-alanine N-methylamide, acetyl-L-valine N-methylamide, acetyl-DL-norleucine N-methylamide and acetyl-L-proline N-methylamide, abbreviated as AGlyMA, A-DL-AlaMA, A-L-ValMA, A-DL-NorlMA, and A-L-ProMA, respectively. The samples are the same as those used in our previous researches.[1,3,5]

The infrared absorption measurements were carried out by a Perkin–Elmer 112G spectrometer with a 75 lines/mm. grating and a KBr fore-prism. The grating is blazed at 12 μ, and its fourth order diffraction was used in the 3 μ region measurements. The wave length calibration for the spectrometer was made with known frequencies of 296 absorption lines reported in a previous paper.[9] The frequency given for any sharp line observed in the present experiment should be accurate up to the figure on the place of 1 cm.$^{-1}$. The effective slit width was made 1.7–2.9 cm.$^{-1}$ (0.2–0.4 mm.) and the resolving power of the spectrometer was more than sufficient for the present purpose.

Dilute carbon tetrachloride solutions of the compounds were placed in a cell of 10 cm. path length, made of fused silica, with plane parallel silica windows on both ends.[10] The cell was heated electrically.

For each sample, measurements were made at 30 and 60°, and at two different concentrations, c_1 and c_2, where c_2 is just half of c_1. The optical path length (l_2) for c_2 was made twice as long as that (l_1) for c_1, so that $c_1 \times l_1 = c_2 \times l_2$ (actually, l_1 = 10 cm. and l_2 = 20 cm.). This was achieved by placing between the source unit and the fore-prism unit of the spectrometer an attachment which consists of two spherical mirrors and two plane mirrors, arranged as shown in Fig. 1. The infrared beam goes forward and backward through the 10 cm. silica cell, and hits the entrance slit with the same direction and with the same aperture as it does without the attachment.

Results and Discussions

The results of the measurements are shown graphically in Fig. 2. For each of the five com-

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