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An X-Ray Scattering Investigation of the Urea Denaturation of Bovine Serum Albumin[1]

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An X-ray scattering investigation of the urea denaturation of bovine serum albumin is described. The protein molecule was studied in the native form, in 6 molar urea and again after dialysis removal of nearly all of the urea, both with regard to external properties (size and shape) and gross internal features. The results of the X-ray work indicate that: the denatured molecule is considerably altered from the native, possessing a 27% larger electronic radius of gyration and a quite disordered internal structure; the denatured protein undergoes an aggregation process involving the sulfhydryl group, which may be prevented by treating the sulfhydryl group of the native protein with *p*-chloromercuribenzoate; in the absence of aggregation effects, the removal of nearly all of the urea by dialysis produces a molecule of size and shape indistinguishable from that of the native molecule; the gross internal features also appear to return, but there is some evidence of slight alteration in tertiary structure.

Introduction

A considerable amount of experimental work has been done in applying X-ray scattering from solutions of proteins and nucleoproteins to the problem of determining the size and shape of these particles. Much of the experimental work and theoretical background is discussed in the recent review article by Beeman, Kaesberg, Anderegg and Webb.[3] In addition to the investigation of the size and shape of macromolecules, X-ray scattering from particles in solution may also be used to investigate their internal structure, although much less detailed information is available than in the case of crystal work because of the random orientation of scattering units.

It is possible then to use X-ray scattering to study changes in external features of a protein molecule in solution upon denaturation and also to obtain some rough information about changes in internal structure. This work constitutes such an investigation of the urea denaturation of bovine serum albumin (BSA).

X-Ray Theory.—In general for a system of N identical and randomly oriented particles which scatter independently, the scattered intensity as a function of angle is given by the expression[4]

$$I(s) = NI_e(s) \int_V \int_V \rho_n \rho_m \frac{\sin sr_{mn}}{sr_{mn}} dv_m dv_n \qquad (1)$$

where $s = 4\pi \sin \theta/\lambda$, λ being the wave length of the radiation and 2θ the scattering angle, I_e is the Thomson scattering intensity for a single electron, ρ_m and ρ_n are the electron densities in the volume elements dv_m and dv_n, respectively, r_{mn} is the separation of the volume elements, and V is the particle volume. Guinier[5] has shown by expanding $\sin sr_{mn}$ that at small angles ($sR < 1$)

$$I(s) = I_e(s) N n^2 e^{-(s^2R^2)/3} \qquad (2)$$

where $R^2 = \frac{\int \rho(r) r^2 dv}{\int \rho(r)\, dv}$, n being the number of electrons in the particle and $\rho(r)$ the electron density a distance r from the center of charge. At small angles then all scattering curves approach a Gaussian of width determined by the parameter R, the electronic radius of gyration of the particle. The angular region for which the Gaussian approximation (equation 2) is valid is called the Guinier region of the scattering curve. For particles in solution n is the excess number of electrons in the particle over the number of electrons in an equal volume of solvent.

A calculation of the form of the scattering curve outside of the Guinier region requires an evaluation of the integral of equation 1, which in turn requires a detailed knowledge of the structure of the particle. Immediately outside of the Guinier region, however, the scattering curve is sensitive predominantly to the external shape of the molecule, and the particles may be treated as if they possessed a constant or slowly varying electron density.

Expressions have been derived for spheres, prolate and oblate ellipsoids, and prolate and oblate cylinders, assuming uniform electron density. Malmon[6] recently has calculated these functions numerically over an intensity range of 10^4. One can then in principle, after correcting for the finite height and width of the X-ray beam, determine the shape of the particle by comparison of observed and calculated scattering curves. In practice the comparison is hampered by the fact that the differences between theoretical curves for different shapes become pronounced only as one considers angles quite far removed from the Guinier region, but at these larger angles the uniform density approximation becomes of dubious validity, and in fact large deviations from any calculated constant density curve arise.

If certain intra-molecular distances predominate within the molecule being studied, the wide angle scattering pattern will show peaks, although the angular position of the peak is not directly related to the predominating distances by Bragg's law, and the use of distances derived from Bragg's law in describing peaks is really a means of classification rather than a statement of a precise parameter of the structure. In order to make a detailed interpretation of peaks, it is then necessary to proceed by comparing the actual scattering curve with the

(1) This investigation was supported in part by a grant from the National Institutes of Health of the United States Public Health Service and in part by the Wisconsin Alumni Research Foundation.

(2) Department of Biophysics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts.

(3) W. W. Beeman, P. J. Kaesberg, J. W. Anderegg and M. B. Webb, "Handbuch der Physik," Vol. XXXII, Springer-Verlag, Berlin, 1957.

(4) P. Debye, *Ann. Phys.* (*Leipzig*), **46**, 809 (1915).

(5) A. Guinier, *Ann. Phys.*, **12**, 161 (1939).

(6) A. G. Malmon, *Acta Cryst.*, **10**, 639 (1957).

scattering calculated on the basis of a particular model.

Arndt and Riley[7] have studied the X-ray powder scattering from a number of globular proteins and noted that all give similar patterns, with two prominent peaks of angular position corresponding to Bragg distances of around 10 and 4.5 Å. The former is interpreted as representing an average spacing between polypeptide chains, and the latter is attributed to the regular folding or coiling of the chain.

In interpreting the angular region around the 4.5 Å. peak, Arndt and Riley make calculations on the basis of the α-helix and other proposed secondary structural configurations. They obtain good agreement between the experimental scattering and that calculated for an α-helix, although the sensitivity of the highly averaged powder scattering patterns in distinguishing one form of secondary structure from another is perhaps open to question. This work at any rate seems to tie this portion of the scattering curve to secondary structure in globular proteins, and it appears reasonable to consider the 4.5 Å. peak as an indication of secondary order in a globular protein, that is order within the polypeptide chain as opposed to the tertiary order of the packing of the chains.

The interpretation of the "10 Å. peak" as a manifestation of the tendency of the coiled polypeptide chains to pack a more or less fixed distance apart is probably on somewhat firmer ground than the consideration of the "4.5 Å. peak" as a helix parameter. Astbury and Street[8] obtained a 9.8 Å. reflection perpendicular to the fiber axis for fibrous proteins, which they interpreted as the spacing between polypeptide chains maintained by side chain interactions—a "packing peak." Kendrew and collaborators[9] have noted the tendency for chains to lie 8–10 Å. apart in their three-dimensional electron density map of myoglobin, and Perutz[10] has pointed out that parts of the three-dimensional vector (Patterson) structure of hemoglobin contain rods spaced approximately 10 Å. apart with maxima at roughly 5 Å. intervals along their length. Arndt and Riley[7] note that a peak of Bragg spacing 10 Å. does not occur in calculations for isolated helices but does occur for a model structure of seven cylindrical shells spaced 11 Å. apart.

In summary, the information available from X-ray scattering by macromolecules in solution which is particularly pertinent to the study of protein denaturation consists of the determination of the radius of gyration (a measure of particle size), an estimate of particle shape in terms of uniform electron density figures of revolution or other charge configurations amenable to calculation, and a rough study of changes in secondary and tertiary structural features from the behavior of peaks in the wide angle scattering pattern.

(7) U. W. Arndt and D. P. Riley, *Proc. Roy. Soc.* (*London*), **A247**, 409 (1955).

(8) W. T. Astbury and A. Street, *ibid.*, **A230**, 75 (1931).

(9) J. C. Kendrew, G. Bodo, H. M. Dintzis, R. G. Parrish, H. Wyckoff and D. C. Phillips, *Nature*, **181**, 662 (1958).

(10) M. F. Perutz, *ibid.*, **167**, 1053 (1951).)

Experimental

The bovine serum albumin was purchased from Pentex, Inc. (Lot numbers 1207 and 9F07). Solutions of "native" BSA were prepared by dissolving the crystalline powder in *p*H 4.7 acetate buffer (0.02 *M* NaAc; 0.02 *M* HAc) and then dialyzing against the same buffer for 24 hr. All dialysis was carried out at 1°. The "denatured" solutions were prepared by dialyzing a native sample against a total of 100 volumes of a solution of the same buffer 6 molar in urea. Dialysis was carried out over a 72 hour period with several changes of bath. "Renatured" solutions were prepared by dialysis of a denatured solution against a total of 400 volumes of the *p*H 4.7 acetate buffer without urea over a 96 hr. period. Solutions for the dilution series of apparent radii of gyration and $I(0)/c$ determinations were prepared by dialyzing an initially 2.5% "native" solution and by making appropriate dilutions from these "master" native, denatured and renatured solutions. The other, more concentrated solutions were all dialyzed separately. Concentrations were determined by ultraviolet absorption at 280 mμ.

The "native PCMB" solution was made up by dissolving the protein in *p*H 9 acetate buffer in the presence of a two times stoichiometric amount of *p*-chloromercuribenzoate (PCMB). The solution was allowed to stand for several hours in the cold and then dialyzed against acetate buffer to bring the *p*H to 4.7 and remove the excess PCMB. The originally alkaline solution was necessary in order to dissolve the PCMB. The "denatured PCMB" and "renatured PCMB" solutions were prepared in the same way as the no PCMB samples. All chemicals used were reagent grade.

The basic X-ray set up has been described previously.[11,12] A rotating water cooled anode is employed in conjunction with a power supply for which current and voltage are electronically stabilized. The incident X-ray beam is defined by a pair of tantalum slits, and the scattered X-ray beam is analyzed by a second pair of slits, rotated about an axis through the center of the sample holder. Copper radiation was employed, with $K\alpha$ monochromatization achieved by a nickel β-filter together with an electronic pulse height analyzer used with proportional counter detection. The solution sample holders were constructed of stainless steel 1 mm. thick with mica windows.

The scattering from the protein was determined by subtracting the background scattering of solvent, sample holder and air from the total scattering. The curves from 5 to 90 milliradians were obtained by combining data for a series of protein concentrations (1, 5 and 12%), since the greater scattered intensity needed at larger angles may be obtained from concentrated solutions, but the concentrations must be kept low in the small angle region to prevent interparticle interference effects from altering the shape of the scattering curve. For the wide angle work a concentration of approximately 15% was used.

Correction for the finite height (here 10 mm.) of the collimating slits was done on an IBM 650 computer, using a program originally written by Anderegg.[13] The slit width correction proved to be negligible with the slit width used (0.6 mm.).

Experimental Results

Size, Shape and Aggregation.—In investigating the external characteristics of BSA in the denaturation process, two sets of experiments were performed. In the first, radii of gyration were determined and extended curves obtained for the protein under the three conditions: native, denatured in 6 molar urea and renatured. Analytical centrifuge runs showed the presence of a large amount of high molecular weight material, presumably aggregate, in both the denatured and renatured samples (Fig. 1). The existence of two ultracentrifuge

(11) B. R. Leonard, Jr., J. W. Anderegg, S. Shulman, P. Kaesberg and W. W. Beeman, *Biochim. Biophys. Acta*, **12**, 499 (1953).

(12) P. Schmidt, P. Kaesberg and W. W. Beeman, *ibid.*, **14**, 1 (1954).

(13) J. W. Anderegg, Ph.D. Thesis, University of Wisconsin, 1952.

peaks in 6 molar urea denatured BSA also has been noted by Gutter, Petersen and Sober.[14] Under these circumstances, it was difficult to distinguish changes in the scattering curve associated with alteration of the protein from changes associated with aggregate formation.

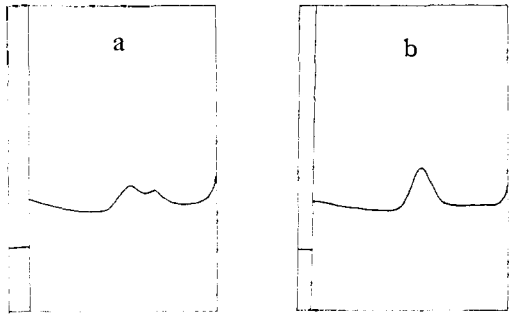

Fig. 1.—Centrifuge diagrams of urea denatured serum albumin: (a) without PCMB; (b) with PCMB.

The second set of experiments was a repeat of the first except that the sulfhydryl groups of the protein molecules were first tied up with the sulfhydryl reagent PCMB. In this case centrifuge runs revealed no aggregate peak—that is, no additional aggregation over that present in the native preparation. (All BSA preparations contain a certain amount of heavy component believed to be dimer.) The success of PCMB in suppressing aggregation is in accord with the work of Huggins, Tapley and Jensen,[15] who have reported success in preventing gelation in concentrated albumin in urea solutions by the use of sulfhydryl reagents.

The use of PCMB thus provided a solution to the aggregation problem. There remained the question of what effect the PCMB might have on the molecule other than reacting with the SH group. In studying the viscosity of urea denatured BSA solutions, Frensdorff, Watson and Kauzmann[16] found a somewhat complex behavior upon the addition of PCMB which they ascribed in part to a catalytic effect of the reagent on the alkaline hydrolysis of S–S bands in the BSA molecule. However, in the native molecule the S–S bands seem to be well buried, since it is necessary to denature the molecule to get at them with oxidizing agents,[17] and so treating the native molecule with PCMB and then dialyzing to remove the excess before denaturation should avoid any problem with S–S rupture. In addition, the experiments were performed at acid *p*H. The X-ray results indicate that the native PCMB molecule probably is identical to the native molecule without the sulfhydryl reagent.

Radii of gyration were obtained from the slope of a log I *vs.* θ^2 plot in accordance with the equation $I = I_e N n^2 e^{-(s^2R^2)/3}$. The apparent radius of gyration was determined for the native, denatured and renatured protein with and without PCMB at four different concentrations—2, 1.5, 1 and 0.5%. The "infinite dilution" value $R(0)$ was then obtained by a straight line extrapolation of a plot of apparent radius of gyration *vs.* concentration. This procedure eliminates interparticle interference effects, which reduce the scattering at the smallest angles.

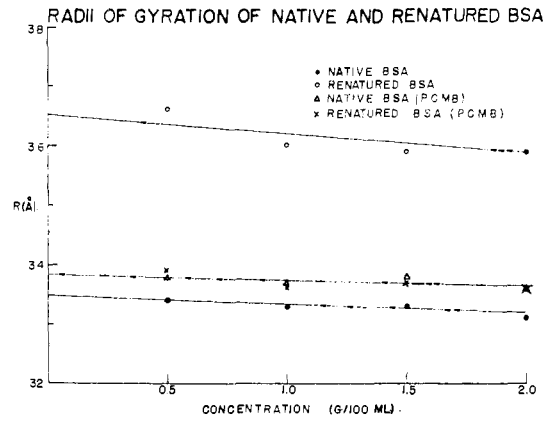

Fig. 2.—Plots of apparent radius of gyration *versus* BSA concentration for the native and renatured material.

It has been shown that the extrapolation should be linear for low concentrations.[18] The plots for the native and renatured samples are shown in Fig. 2 and for the two denatured samples in Fig. 3. Values of $R(0)$ in Å. are summarized in Table I below. The error in the determination is about 1% for the native and renatured samples and about 3% for the denatured samples.

TABLE I

RADIUS OF GYRATION OF BSA AFTER VARIOUS TREATMENTS IN Å.

	Native	Denatured	Renatured
No PCMB	33.5	46.2	38.0
PCMB	33.8	41.6	33.8

As judged by the radius of gyration criterion, the native and renatured samples of the PCMB series appear identical. The slight difference between the native protein with and without PCMB (if real) may result from the presence of the high electron density mercury atom. As would be expected from the evidence of aggregation in analytical centrifuge runs, the renatured (without PCMB) sample possesses a considerably larger radius of gyration. The denatured protein is clearly more spatially extended than the native. The denatured material without PCMB possesses a considerably larger radius of gyration than its PCMB counterpart, which is consistent with the presence of the aggregate peak in the analytical centrifuge pattern.

The existence of aggregation effects in the renatured sample without PCMB is also revealed by a consideration of the scattered intensity at zero angle. The ratio of the zero angle scattering to the con-

(14) F. J. Gutter, E. A. Petersen and E. A. Sober, *Arch. Biochem. Biophys.*, **73**, 194 (1957).

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

(16) H. K. Frensdorff, M. T. Watson and W. Kauzmann, THIS JOURNAL, **75**, 5167 (1953).

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

(18) G. Fournet, *Compt. rend.*, **228**, 1421 (1949a).

centration is proportional to the average molecular weight. Although absolute intensity measurements are necessary in order to evaluate the absolute molecular weight, it is possible to determine relative molecular weight from a relative $I(0)$ determined by an extrapolation of the Guinier log I *vs.* θ^2 plot, providing solvent composition and sample holder transmission are maintained constant, and adjustment is made for intensity differences in the primary (incident) beam. The values of $I(0)/c$ (in relative units) extrapolated to infinite dilution were between 65 and 67 for the native, native PCMB and renatured PCMB samples and 78 for the renatured (no PCMB) sample, showing a relative molecular weight difference and the presence of aggregation. Values of $I(0)/c$ for the denatured samples are not comparable because of the increased electron density of the solvent due to the presence of the urea.

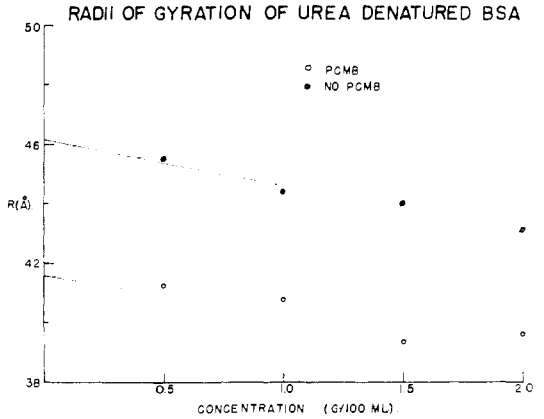

Fig. 3.—Plots of apparent radius of gyration *versus* BSA concentration for urea denatured material, with and without PCMB.

The radius of gyration results indicate a considerably distended, denatured molecule which is, however, able to renature to its original size, providing aggregation is prevented. The extended curves indicate a similar return of the original particle shape and also indicate identity of native and native PCMB molecules. Figures 4 and 5 show the close identity of the scattering curves for the native, native PCMB and renatured PCMB samples. Figure 4 shows the intensity *vs.* angle plot of the data taken for the native and native PCMB samples over the angular range 5 to 90 milliradians (mr.). Experimental points shown are alternately native and native PCMB. The agreement is complete over the region sensitive to particle shape (roughly 15 to 65 mr.) and includes the internal structural peak at 75 mr. Figure 5 shows a similar plot for the native and renatured samples of the PCMB series, again alternating experimental point. The excellent agreement of the two curves over the angular range sensitive to particle shapes indicates a complete return of the original shape in the renatured particle. The peak region will be discussed in the next section.

Figure 6 shows the slit corrected scattering curves for the native and denatured PCMB molecules, plotted against sR rather than the angle, a procedure which eliminates the difference in radius of gyration (since all curves go as $e^{-(s^2R^2)/3}$ at the smallest angles) and facilitates comparison with theoretical curves. The numbered marks indicate the points through which theoretical curves for uniform electron density prolate and oblate ellipsoids pass. Actually neither curve follows a theoretical counterpart over a large enough angular range to really prove a case for a precise shape, but there is evidence for greater particle asymmetry in the denatured sample on a uniform ellipsoid interpretation.

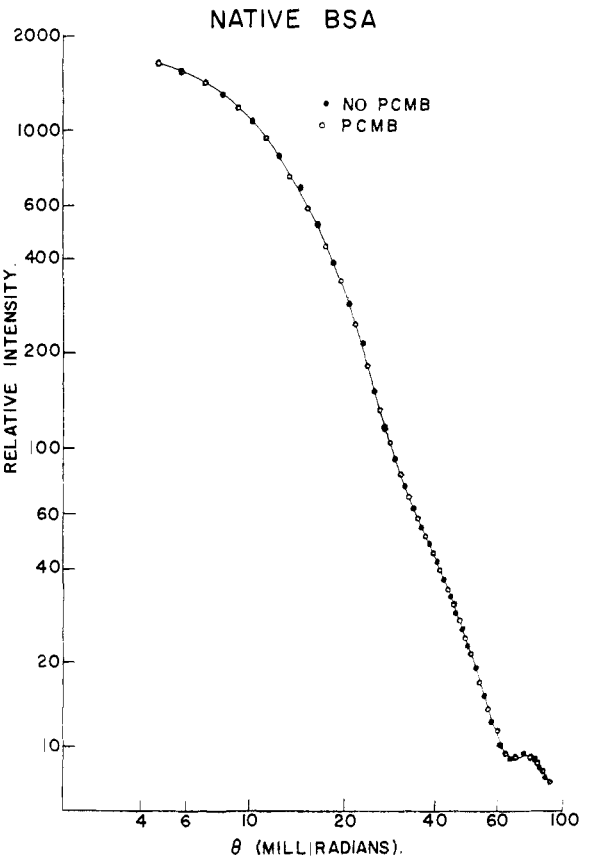

Fig. 4.—The intensity of scattered X-rays as a function of angle from BSA solutions with and without PCMB. The nearly identical size and shape of the molecules is illustrated.

If urea denaturation is predominantly a helix-random coil transition, as has been inferred by Yang and Doty[19] on the basis of optical rotation work with proteins and synthetic polypeptides, the state of the denatured molecule would be expected to consist of some helix (since there appear to be further physical changes at higher urea concentration)[20] interspersed with larger regions of disordered coiling, which cannot, however, be completely random because of the 16 S–S links still intact to hold the molecule to some degree of globular form. A comparison of the urea scattering curve to that of a random coil is then perhaps as valid as comparison to a uniform ellipsoid of revolu-

(19) J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957).
(20) H. Neurath and A. M. Saum, *J. Biol. Chem.*, **128**, 347 (1939).

tion, although neither model would be expected to be correct. The urea curve does in fact exhibit a roughly $1/\theta^2$ region at the larger angles, which is the asymptotic angular dependence of the scattering from a random coil for $sR>>1$. However, scattering from a "random coil" synthetic polypeptide (poly-benzyl-glutamate in hydrazine) showed no such $1/\theta^2$ region, and so this agreement is perhaps fortuitous. It is clear then from the extended curve in the shape sensitive region that the denatured molecules have undergone a large alteration, but a clear-cut separation of changes involving greater asymmetry from changes involving transitions to disordered coiling does not seem feasible.

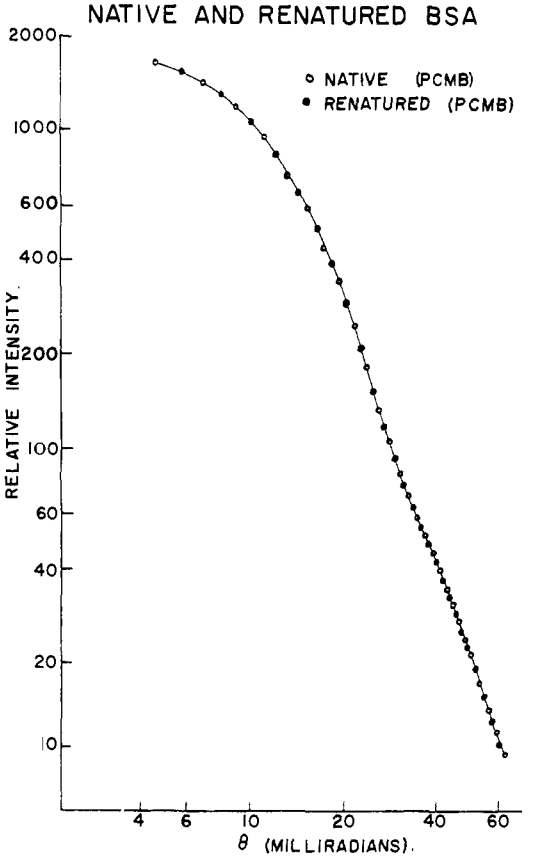

Fig. 5.—The intensity of scattered X-rays as a function of angle from solutions of native and renatured BSA, both with PCMB. The nearly identical size and shape of the molecules is illustrated.

The effect of aggregation on the extended portions of the scattering curve throws some light on the nature of the aggregate. If an aggregation is "loose" enough so that each particle retains in a large measure its own structural identity, the aggregation process would be expected to manifest itself as a type of interparticle interference effect, producing the largest effect at the smallest angles (*i.e.* on the radius of gyration) and a progressively smaller effect at larger and larger angles, so that over the outer portions of the shape sensitive angular region the scattering should be characteristic of the monomer. Physically, this is merely a statement that the scattering at larger and larger angles is sensitive to smaller and smaller distances. A mathematical treatment of interparticle effects has been given by Fournet.[18] The albumin aggregation in the denatured and renatured solutions without PCMB produces essentially no difference in the scattering curve past 25 milliradians from the PCMB samples, and the aggregation hence does not appear to involve any gross changes in the molecular properties of the monomer.

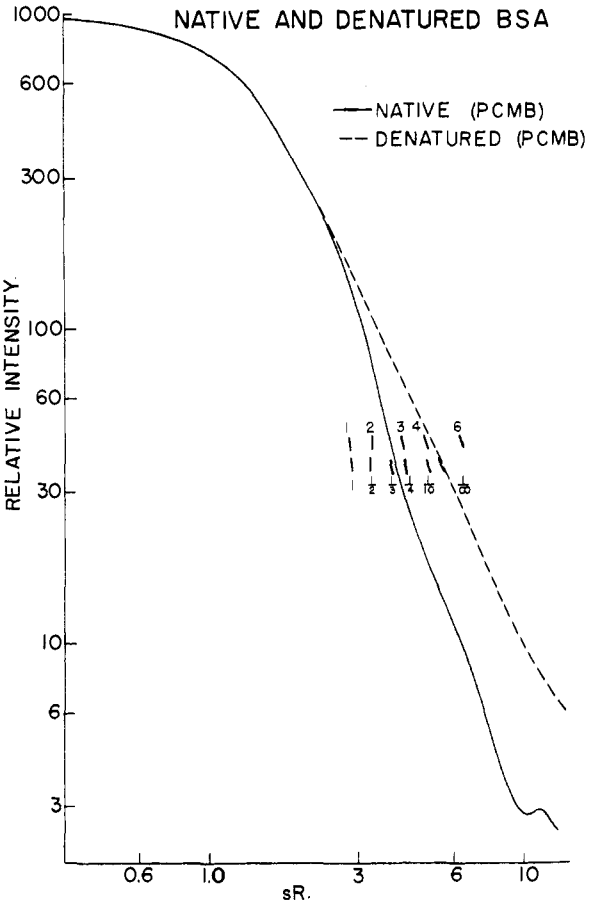

Fig. 6.—The intensity of scattered X-rays as a function of sR from solutions of native and denatured BSA, both with PCMB. The denatured molecule has clearly altered. It is not possible to distinguish between a change in axial ratio and symmetrical unfolding.

Peak Region and Internal Structure.—In discussing the results of the wide angle scattering patterns in the angular regions sensitive to internal structure, the "10 Å. peak" will be considered as a "packing peak," representing an average spacing between coiled polypeptide chains and therefore a measure of tertiary structural order. The "4.5 Å. peak" will be considered as an indication of secondary structure or a "coiling peak." It should be emphasized that this assignment of peaks is merely an interpretation and has not been conclusively established but it appears a very plausible interpretation. There is a third peak observed in solution with a maximum around 4.2°, corresponding to a Bragg spacing of 20.5 Å. The interpretation of this peak will be discussed later.

It should be noted that there are definite differences in the wide angle scattering curves for native serum albumin when dry and when in solution. Figure 7 compares the scattering curves for a sample in acetate buffer and a freeze dried sample. The packing peak maximum in the dry sample is at 9.2° (or 9.7 Å. in spacing terms) but in solution shifts to 7.6° (11.6 Å.) with a remnant shoulder where the dry maximum existed. An appealing explanation

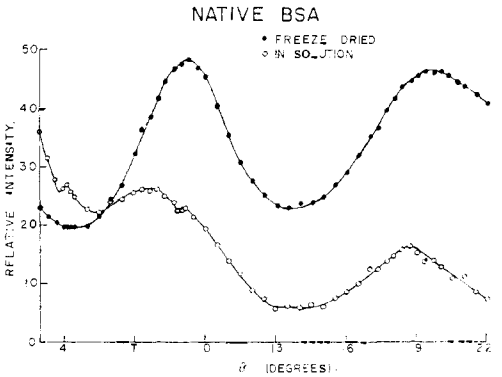

Fig. 7.—The X-ray scattering at moderately large angles from freeze dried BSA and a solution of BSA.

is that the penetration of solvent molecules has produced a separation of polypeptide chains, except perhaps where the distance of separation is fixed by S-S bonds (which might explain the shoulder at the dry maximum). This would produce a greater wet volume for the molecule than dry, a phenomenon observed for BSA,[21] catalase[22] and spherical plant viruses.[1] However, a less appealing but possibly more likely explanation is that this peak shift results from the fact that in scattering from the albumin powder there are very strong interparticle interference effects which produce a greatly depressed small angle scattering. Therefore, the dry peak is superimposed on a curve which is nearly flat or even sloping slightly up with angle, whereas the solution scattering peak is superimposed on a curve which is still falling off with angle. This indicates the difficulty in interpreting peaks in terms of definite distances within the molecule. There also appears a slight shift (from 4.6 to 4.8 Å. in spacing terms) in the coiling peak, but a peak in the water scattering in this angular region produces a high and steeply rising solvent background and makes the accurate determination of the shape of this peak quite difficult, and hence this difference is also of doubtful significance.

Figure 8 shows the scattering curves obtained from solutions of native and denatured BSA with PCMB added. Only a semblance of the packing peak remains in the denatured material, implying a considerable breakdown of tertiary structural order. This behavior seems to imply the disruption of weak tertiary bonds in urea denaturation, although it could arise because transition of secondary helical structure to a more random state creates a greater randomization of inter-chain distances. However, the widely varying rates of urea denaturation with different proteins[23] indicate that the process cannot be completely interpreted as a simple helix-random coil transition of the secondary structure, although breakdown of the hydrogen

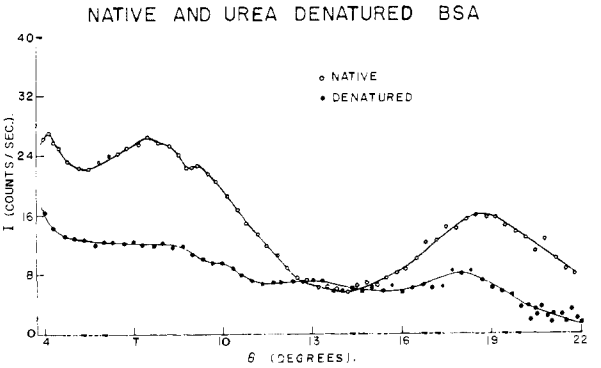

Fig. 8.—The X-ray scattering at moderately large angles from solutions of native and urea denatured BSA, both with PCMB.

bonded secondary structure of course should be important. The existence of this breakdown of secondary structure is supported by the alteration of the coiling peak in the scattering from the denatured protein.

Figure 9 presents the scattering curves for the native and renatured PCMB samples, normalized to the same intensity at 5.5 degrees for purposes of

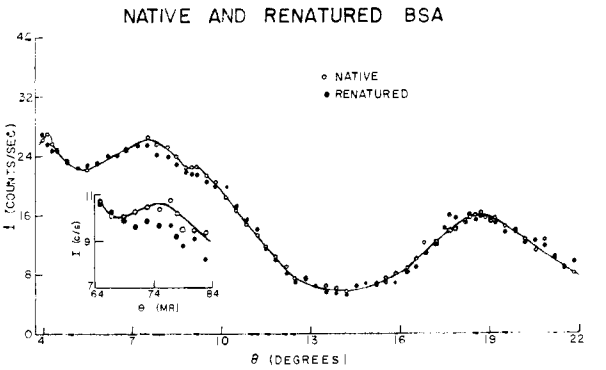

Fig. 9.—The X-ray scattering at moderately large angles from native and renatured BSA solutions, both with PCMB.

comparison. The general features of the internal structure of the renatured molecule appear to be the same as the native, judging from the quite good agreement of the two curves. It appears that the polypeptide chains tend to recoil and the tertiary structure to reform when the denaturing agent is removed. In the packing peak, however, which is much the more amenable to experimental study in solution, there does appear to be a difference slightly greater than the experimental error. The estimated error in the sample counting rate in this an-

(21) J. W. Anderegg, W. W. Beeman, S. Shulman and P. Kaesberg, THIS JOURNAL, **77**, 2927 (1955).

(22) A. G. Malmon, *Biochim. Biophys. Acta*, **26**, 233 (1957).

(23) W. Kauzmann and R. B. Simpson, THIS JOURNAL, **75**, 5154 (1953).

gular region is of the order of 3% or about $^3/_4$ count per second, so that the consistent 1–1.5 count per second difference observed between 8 and $9^1/_2$ degrees is perhaps meaningful and may imply slight alteration in the juxtaposition of coiled polypeptide chains. The agreement of coiling peaks is better although the estimated error is around 9% and the curve is much more subject to shape distortion because of the steeply rising background which must be subtracted.

In considering the peak region of the scattering curve, only passing mention has been made so far of the "20 Å. peak," primarily because its origin seems at present quite unclear, and therefore its usefulness in interpreting internal structural behavior is limited. The peak can be seen in Fig. 9 as a small blip at 4.2° in the native sample scattering and is shown in the inset in more detail. The peak disappears in urea and as may be seen does not completely return on renaturation. It then represents some aspect of structure (presumably tertiary) which does not completely renature. Arndt and Riley[7] find a small peak corresponding to a "half-order reflection" or a "next-nearest neighbor distance" in calculations for their model system and mention observing such a peak with several proteins. Another explanation for such a peak might be that it represents a spacing between structural subunits.

Discussion

The X-ray results of scattering from bovine serum albumin indicate that the denaturation produces a considerably altered molecule but that the process is completely reversible as far as the external features of the molecule are concerned, provided aggregation effects are prevented by the use of the sulfhydryl reagent PCMB. The size and shape of the renatured molecule is completely indistinguishable from the native. The presence of the second ultracentrifuge component (the presumed dimer) in the native preparation constitutes an annoyance but should not affect any of the conclusions, provided the amount of dimer present does not change in the renatured sample. As nearly as can be judged from the centrifuge peaks, the amount of dimer was identical in the native and renatured samples. Even if the amount did change, the effect would probably only show up in the radius of gyration determination. For two "native" samples with radii of gyration of 33.5 and 30 Å., the difference in scattering curves was confined to angles inside of 15 milliradians, indicating that the extra material probably is dimer and that dimerization is loose enough to preserve the structural identity of the particle (since the shape sensitive region of the scattering curve was unaffected).

The value of 33.5 Å. for the radius of gyration of the native BSA molecule is considerably larger than the 29.8 Å. value of Anderegg[21] and the 30.2 Å. value of Rothwell.[24] Champagne, Luzzati and Nicolaieff[25] have obtained an intermediate value of 31.5 A. The discrepancy apparently reflects a difference in dimer content of the native preparation used in the different investigations. The sample used in the present investigation possessed a larger than typical amount of fast component in the analytical centrifuge pattern, consistent with the high radius of gyration.

As far as internal features of the molecule are concerned, the general structural organization also appears to be a property of the solution conditions. The scattering curve in the peak region is greatly altered in urea but tends to resemble the original native curve quite well on removing the urea, so that no matter what interpretation is placed on the peaks, the conclusion of disordering of structure in urea and general tendency to return will be valid. If the more detailed interpretations with respect to structure are applied to the peaks, one would say that denaturation is characterized by considerable disruption of both secondary and tertiary structure and a subsequent return of the basic structural features on renaturation. This is consistent with a view of urea denaturation as a randomization of secondary structure produced by breaking of structural hydrogen bonds, coinciding with a loss of weak tertiary bonding.

The question as to whether renaturation is complete is a difficult one. Even identity of peak regions would not prove identity of structure because of the tremendous averaging which occurs in scattering from randomly oriented materials. On the basis of differences which do exist in the scattering curves and subject to the interpretations used for the peaks, it appears that secondary structure may completely reform (as judged by the 4.5 Å. peak) but that the tertiary structure suffers some slight alteration.

This viewpoint of a completely reversed secondary structure is consistent with the reversibility of specific optical rotation noted by Aten, *et al.*, on dialysis removal of urea[26] and by Kauzmann and Simpson on dilution of urea,[23] on the basis of optical rotatory power as a measure of secondary coiling. Tertiary alterations would explain the changes in susceptibility to tryptic fission and capacity to produce antibody noted by Neurath, Cooper and Erickson[27] for their "regenerated" horse serum albumin and Putnam and co-workers[28] with BSA, even though the molecular kinetic properties of diffusion and viscosity were essentially the same as in the native material. These workers separated a "regenerated" material from an "irreversibly denatured" material after removal of urea by dialysis and fractionation by heating or salting out. It may be that the "irreversibly denatured" material corresponded to that portion of the protein sample which had undergone aggregation. From this viewpoint it is interesting that Putnam, *et al.*, find 50% irreversibly denatured with crystalline BSA—approximately the magnitude of the aggregate centrifuge peak in the present investigation.

The subject of urea denaturation of serum albumin has been a source of some controversy, chiefly

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with regard to aggregation effects and the influence of urea on particle shape. Earlier measurements are discussed by Putnam[29] and generally concur in finding the same molecular weight for the protein in urea as in the native state. However, Putnam[29] mentions a diffuse boundary in sedimentation in 8 molar urea, indicating heterogeneity. Charlwood[30] notes a similar effect in 8 molar urea at *p*H 9.9 which does not appear when PCMB is used. Frensdorff, *et al.*,[16] and Hospelhorn, *et al.*,[31] infer aggregation on the basis of viscosity measurements. Gutter, Petersen and Sober,[14] as mentioned earlier, observe sedimentation evidence of aggregation in 3–6 molar urea at *p*H 4.5 but not in 7.5 molar. Kay and Edsall[32] find no aggregation in bovine mercaptalbumin in 8 molar urea at *p*H 4.75 as judged by the concentration to turbidity ratio $(c/\tau)\ c_{\to 0}$. However, the gelation of urea solutions of concentrated BSA[15] appears to be clear evidence that aggregation does occur under some conditions.

The general trend of recent investigations seems to point to the conclusion that BSA does aggregate in concentrated urea solutions, but the extent and rapidity are strongly dependent on the factors of *p*H, protein concentration, urea concentration and method of solution preparation, and the effect may be very small under certain conditions. High *p*H and high protein concentration appear to favor aggregation.[33] It is therefore necessary to check carefully the aggregation situation before drawing conclusions based entirely on intra-molecular behavior.

Diverging views also exist concerning particle shape. Neurath and Saum[20] interpret their diffusion and viscosity measurements in terms of an axial ratio increase with urea concentration from 4.3 to 19.4 in going from 0 to 8 molar urea. This view of denaturation as an unfolding of the globular, compact native state to a very asymmetric one found support in the work of Astbury, Dickinson and Bailey,[34] who succeeded in producing macroscopic fibers from urea denatured edestin and a β-type X-ray fiber pattern from rolled films dried from this solution.

Scheraga and Mandelkern[35] have applied their concept of "an effective hydrodynamic ellipsoid," characterized by the function β determined from kinetic properties, to the data of Neurath and Saum. They conclude that the denaturation process involves an increased effective volume due to swelling without appreciable change of axial ratio. The fact that the values of β in urea solution were less than the theoretical minimum is a somewhat disturbing note.

More recently Charlwood[30] has used this interpretation in terms of the β-function in considering sedimentation and viscosity measurements on denatured and renatured BSA in the presence of PCMB. He finds an initial axial ratio increase in urea (from 2.6 to 4.6) followed by a subsequent time dependent increase (from 4.6 to 9.2) which he interprets as resulting from the PCMB catalysis of alkaline hydrolysis of S–S bonds suggested by Frensdorff, *et al.*, and mentioned earlier. Charlwood's renatured particle is quite close to the native in kinetic properties, with intrinsic viscosity of 0.041 renatured *vs.* 0.042 native and sedimentation coefficient of 4.6*S* to 4.4*S*. However, according to the method of Scheraga and Mandelkern (and assuming identity of molecular weights) this corresponds to a big change in axial ratio to 5.2, since β is a very sensitive function of axial ratio. This change is in disagreement with what is observed by X-ray scattering. However, the experimental situations differed in that in the X-ray work the SH groups were tied up with PCMB before the denaturation experiment and the excess removed by dialysis.

On the basis of light scattering, Doty and Katz[36] conclude that urea denaturation of serum albumin produces an approximately isotropic swelling, together with large binding effects. Kay and Edsall[32] concur in later light scattering work with bovine mercaptalbumin. They also note the denaturation appears reversible between 8 molar and 1 molar urea as judged by equivalence of concentration turbidity ratio $(c/\tau)\ c_{\to 0}$, interaction parameter λ obtained from the shape of the c/τ *vs.* c extrapolation, dissymmetry ratio and rate of formation of mercury dimer.

Kauzmann[37] has pointed out that a comparison of molecular kinetic properties of denatured proteins to those of a randomly coiled polymer might be more appropriate than to an ellipsoid of revolution and noted that the intrinsic viscosity of denatured BSA in which the S–S bonds have been broken is roughly comparable to random polymers of the same molecular weight. However, this is not true of denatured serum albumin with S–S links intact, which emphasizes the point made earlier, in considering scattering curve comparison with theoretical curves, that for urea denatured BSA neither of the limiting viewpoints of ellipsoid of revolution of constant electron density nor random polymer has much validity.

The technique of X-ray scattering from solutions of proteins appears a sensitive and useful one in studying the denaturation process. It provides a convenient measure of gross external deformation and may be used as a rough probe of internal disordering. The technique should be particularly useful in investigating reversibility of denaturation because it provides a three-fold characterization of the native (and renatured) protein: size, shape and gross internal features. In the latter capacity, as a way of investigating tertiary structural order, X-ray scattering can provide a useful complement to optical rotation, which has come to be considered as a measure of secondary structural order.

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The Radiation Induced *cis–trans* Isomerization of Polybutadiene. III[1]

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A study was made of the γ-ray induced *cis–trans* isomerization of polybutadiene in solution and in the solid state, in the absence of any deliberately added sensitizer. Starting with an essentially all-*cis* or all-*trans* polybutadiene, the "unsensitized" isomerization approaches a radiostationary equilibrium in which the *cis/trans* ratio in the polymer is about 20/80, in contrast to the thermodynamic equilibrium ratio of 8/92 obtained in previous sensitized isomerizations of polybutadiene. The unsensitized reaction is first order in *cis* (or *trans*) content with a rate which is proportional to the radiation intensity. The isomerization in a 1% solution in benzene is about eight times as fast as that in the pure polymer. The G_0 values for the *cis* → *trans* reaction, *i.e.*, the number of *cis* double bonds initially converted to *trans* per 100 e.v. of energy absorbed by the polymer and the solvent, in the solution case, and by the polymer alone, in the solid state case, are about 1.2 and 14.6, respectively. The corresponding G'_0 values for the *trans* → *cis* reaction are about 0.3 and 3.6, respectively. The mechanism of the unsensitized isomerization is believed to involve excitation of the π electrons of the polymer double bonds to an antibonding state where free rotation, and hence geometric interconversion, can occur. This excitation, in the solid state, presumably results from collisions of energetic electrons with the polymer molecules: the double bond electrons are excited directly and/or indirectly through intramolecular energy transfer from the methylenic groups in the polymer. These processes occur also in solution but are less important there than intermolecular energy transfer from excited or ionized solvent molecules to the polymer double bonds.

Introduction

The radiation induced *cis–trans* isomerization of polybutadiene in benzene sensitized by bromine atoms or thiyl radicals was described in two previous papers.[2,3] The existence of an "unsensitized" isomerization in solution, *i.e.*, where no particular sensitizer was deliberately added to the polybutadiene prior to γ-irradiation, was briefly mentioned. It also was pointed out[2] that no evidence had yet been found for an analogous isomerization of the pure polymer in the solid state. Such a reaction could be expected on the basis of possible direct excitation of the polymer molecules, and hence also the π electrons of the double bonds, through impact with the Compton and secondary electrons generated by the γ-rays. Since the unsensitized isomerization of polybutadiene was considered to be a potentially very useful reaction for investigating energy transfer processes in irradiated polymers, it was desirable to re-examine the solid state irradiation of polybutadiene and at the same time obtain further data on the unsensitized isomerization in solution.

Recently it has been found that the pure polybutadiene can indeed undergo isomerization on γ-irradiation in the solid state. The rate is only about one-eighth of that in the unsensitized solution case which is itself very slow compared to the sensitized isomerizations considered previously. Nevertheless, the unsensitized isomerization, in solution or in the solid state, turns out to be a highly efficient non-chain reaction when viewed from the standpoint of energy utilization. Furthermore, both of these unsensitized isomerizations, starting from either the all-*cis* or all-*trans* polybutadiene, approach a radiostationary equilibrium in which the *cis/trans* ratio is about 20/80, in contrast to the thermodynamic equilibrium value of about 8/92 obtained in the sensitized isomerizations. This clearly indicated a fundamental difference between the mechanisms of the sensitized and unsensitized isomerizations, quite apart from the role of the sensitizer itself. The present work is therefore concerned also with elucidating this difference, which presumably is due to the sensitized isomerization proceeding through a transitory radical adduct whereas the unsensitized isomerization must involve an excited state of the polymer double bond.

Experimental

Two polybutadiene samples were used in this work, one having the monomer units arranged in a nearly all-*cis* configuration (CPB) and the other in a nearly all-*trans* configuration (TPB). The polymers were prepared by stereospecific polymerization techniques to a viscosity average molecular weight of around 400,000 for the *cis* polymer and around 150,000 for the *trans* polymer. The structures of the polybutadienes, as determined by infrared analysis, consisted of approximately 95% *cis*-1,4 and 2% *trans*-1,4 units in CPB, and about 95% *trans*-1,4 and 2% *cis*-1,4 units in TPB, with the remaining unsaturation in either polymer made up of vinyl groups resulting from 1,2-addition polymerization. Since the 1,2 units were not involved in the isomerization, only the percentage of the total 1,4 polymerization units having the *cis* (or *trans*) configuration, or, alternatively, the *cis/trans* ratio, in the polymer before and after irradiation was required. The *cis/trans* ratios of CPB and TPB were thus taken to be 98/2 and 2/98, respectively.

Stock solutions of CPB in benzene, with and without antioxidant, and of TPB in benzene-d_6, without antioxidant, were prepared for γ-irradiation to a polymer concentration of about 10 g./l. Additional solutions of CPB in benzene at concentrations up to about 18 g./l. as well as solutions of this polymer in toluene, *m*-xylene, ethylbenzene and tetralin, also were employed. It was necessary to carry out the solution isomerization in an aromatic solvent since the polybutadiene gels on γ-irradiation in aliphatic, cycloaliphatic or other solvents and thus cannot be recovered in a form suitable for infrared analysis. However, on irradiation in benzene or toluene, the polymer undergoes considerable phenylation which, while not affecting the isomeri-

(1) A portion of this material was presented at the 125th Meeting of the American Association for the Advancement of Science, Washington, D. C., December, 1958. Work was accomplished under Air Force contract, the sponsoring agency being the Materials Laboratory of Wright Air Development Center.

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