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An Investigation of the Size, Shape and Hydration of Serum Albumin by Small-angle X-Ray Scattering[1]

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RECEIVED DECEMBER 30, 1954

A study has been made of the X-ray scattering at small angles from solutions of bovine serum albumin, human mercaptalbumin and mercaptalbumin-mercury dimer. The data furnish the radius of gyration of each molecule and assist in the determination of shape and hydration. Radii of gyration of 29.8 Å. for BSA, 31.0 Å. for HMA, and 37.2 Å. for the HMA dimer are obtained. Assuming an ellipsoid of revolution as a model an axial ratio of $(3.5)^{-1}$ oblate is found to be in best agreement with the extended scattering curve. Comparing this with known data on BSA implies an internal hydration of 0.37 and an external hydration of 0.11 g. of water per g. of protein. The ellipsoid of revolution, however, is in disagreement with the single-crystal work on human serum albumin. Models reasonably consistent with both the small-angle scattering and single-crystal work are a modified right prism having dimensions of 106 × 21.5 × 50 Å. and no internal hydration and a rectangular parallelepiped having dimensions of 82.5 × 27.5 × 63 Å. and an internal hydration of 0.59. A best molecular size and shape cannot be chosen at present. It is noted that there is considerable similarity among the ellipsoid, prism and parallelepiped models. A careful discussion of instrumental corrections to the data and of interparticle interference effects is given.

Introduction

Recent experimental work[3-8] has shown that small angle X-ray scattering is a useful method for studying the size and shape of protein molecules in solution. Information concerning their hydration can also be obtained by a comparison of the X-ray measurements with the results of sedimentation, diffusion and viscosity studies. The latter measurements are influenced by water bound to the outside of the molecules while the X-ray measurements are independent of it.

In this paper we present a detailed study of the small angle scattering from solutions of a single protein. We have studied the scattering under different conditions of solutions and over as large an angular range as possible with careful consideration given to the various factors influencing the results, such as background scattering, the finite size of the collimating slits and interparticle interference effects.

Serum albumin was chosen for this investigation because of the large amount of information concerning it in the literature and because it is available commercially in purified form. We have studied the scattering from solutions of bovine serum albumin (BSA), the mercaptalbumin fraction of human serum albumin (HMA), and a dimer of the mercaptalbumin molecule.

Theory

The intensity of the X-rays scattered from a collection of identical particles whose positions and orientations are completely random is given by the expression

$$I(h) = I_e(h)N\overline{F^2(h)} \qquad (1)$$

where $h = 4\pi\lambda^{-1}\sin\theta/2$, θ is the angle of scattering, λ the wave length of the X-rays, $I_e(h)$ the intensity scattered by a single electron, N the number of particles in the scattering volume, and $\overline{F^2(h)}$ is the average over all orientations of the square of the structure factor for the particles. The structure factor of a particle depends on its size and shape, the internal charge distribution, and on the orientation of the particle with respect to the incident X-rays. The structure factor at $h = 0$ will be equal to the total number, n, of electrons in the particle and hence we can write in place of eq. 1

$$I(h) = I_e(h)Nn^2P(h) \qquad (2)$$

where $P(h)$ is a scattering function normalized to unity at zero scattering angle.

Because of destructive interference between waves scattered from different parts of the same particle, $P(h)$ will be small compared to unity at scattering angles greater than $\theta = \lambda/d$ where d is some average linear dimension of the scattering particle. Thus, for proteins with dimensions in the neighborhood of 100 Å. and CuKα radiation with a wave length of 1.54 Å., the strong scattering will be chiefly at angles smaller than 0.015 radians or about 1°. At these small angles $I_e(h)$ is essentially constant and the angular dependence of the scattered intensity is given entirely by $P(h)$. For particles in solution n will be the number of electrons in the particle minus the number of electrons in an equal volume of the solvent. Since the electron density of proteins is approximately 4/3 that of water, the presence of the solvent reduces the scattering by a factor of about sixteen. For our purposes it is not necessary to know the actual number of excess electrons since only relative intensities as a function of angles are involved in the work to follow.

The scattering function $P(h)$ has been calculated by various authors[9,10] for certain simple cases such as spheres, ellipsoids of revolution, cylinders and prisms, all of uniform electron density. For uniform spheres of radius a, $P(h)$ is given by

$$P(h) = \Phi^2(ha) = \left[3\,\frac{\sin(ha) - ha\cos(ha)}{(ha)^3}\right]^2 = \frac{9\pi}{2}\left[\frac{J_{3/2}(ha)}{(ha)^{3/2}}\right]^2 \qquad (3)$$

(1) Supported by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, by the Office of Naval Research, and by the Public Health Service.

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(3) O. Kratky, *J. Polymer Sci.*, **3**, 195 (1948).

(4) D. G. Dervichian, G. Fournet and A. Guinier, *Bull. soc. chim. Biol.*, **31**, 101 (1949).

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(7) B. R. Leonard, Jr., J. W. Anderegg, S. Shulman, P. Kaesberg and W. W. Beeman, *ibid.*, **12**, 499 (1953).

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

(9) A. Guinier, *Ann. Physik*, **12**, 161 (1939).

(10) G. Porod, *Acta Phys. Austriaca*, **2**, 255 (1948).

For uniform ellipsoids of revolution with semi-minor axis a, semimajor axis b, and axial ratio $v = b/a$, $P(h)$ is given by

$$P(h) = \int_0^{\pi/2} \Phi^2(ha\sqrt{\cos^2\theta + v^2 \sin^2\theta}) \cos\theta \, d\theta \quad (4)$$

Guinier[9] has shown that for a particle of arbitrary shape, the scattering at sufficiently small angles approaches the Gaussian

$$P(h) = e^{-h^2R^2/3} \quad (5)$$

where R is the radius of gyration of the charge distribution of the particle about the center of charge. R is defined by the relation

$$R^2 = \frac{\int_v \rho(r) r^2 dv}{\int_v \rho(r) dv} \quad (6)$$

$\rho(r)$ is the electron density in the volume element dv and r is the distance of this volume element from the center of charge of the particle. The integration is extended over the volume of the particle. Thus by plotting the logarithm of the scattered intensity against the square of the scattering angle a straight line should be obtained at very small angles, the slope of which determines the radius of gyration. The radius of gyration may be determined independent of any assumptions about the shape or structure of the particle. Additional information about the size and shape of the particle can be obtained by comparing the complete scattering curve with calculated scattering functions, $P(h)$, such as those given above.

Because of the low intensity of the scattering from protein solutions and the fine collimation needed in order to work at small angles it is seldom possible to use protein concentrations low enough to adequately satisfy the requirement of random positions of the scattering particles. In this case eq. 1 is no longer a good approximation and a theory is required which takes into account the interference between waves scattered from neighboring particles. This problem was first discussed by Zernicke and Prins[11] for the case of spherical particles and was extended to particles of general shape by Mencke[12] and Fournet.[13] For the case where the orientation of the scattering particles is completely independent of their relative positions they obtain the result

$$I(h) = I_e(h) N \overline{F^2(h)} \left\{ 1 - F_1(h) v_1^{-1} \int_0^\infty [1 - W(r)] \frac{\sin(hr)}{hr} 4\pi r^2 dr \right\} \quad (7)$$

The distribution function $W(r)$ is defined so that the probability of finding a particle in an element of volume dv_k and at the same time finding another particle in the element dv_j at a distance r from dv_k is $W(r)\, dv_k dv_j / v_1^2$ where v_1 is the average volume available to each particle ($v_1 = M/cN_0$, M is the molecular weight of particle, c is the concentration in g./ml., N_0 is Avogadro's number). The factor $F_1(h) = \overline{F(h)}^2/\overline{F^2(h)}$ is the ratio of the square of the average (over all orientations) of the structure factor to the average of the square of the structure factor. The average of the square of the structure factor can be determined experimentally but the square of the average structure factor must be calculated. In order to make this calculation a knowledge of the structure of the particle is needed. For particles with spherical symmetry $F_1(h)$ is identically equal to unity.

Using the Born and Green theory of fluids, Fournet[13] has derived an alternative expression for the scattering from a system of particles which gives the scattered intensity in terms of the interparticle force potential, $\Phi(r)$, and the temperature, T, rather than in terms of a purely geometric distribution function. This expression is

$$I(h) = I_e(h) N \overline{F^2(h)} \left\{ 1 + F_1(h) \frac{\epsilon\beta(h)}{v_1(2\pi)^{-3/2} - \epsilon\beta(h)} \right\} \quad (8)$$

Here $\beta(h)$ is defined by

$$h\beta(h) = \frac{1}{2\pi} \int_{-\infty}^{\infty} (e^{-\Phi(r)/kT} - 1) \, r \sin(hr) \, dr \quad (9)$$

ϵ is a constant generally nearly equal to unity.

This expression is convenient for following the change in the shape of the scattering curve with increasing concentration. In very dilute solutions it reduces to eq. 1. As the concentration increases eq. 8 shows that the effect of the interparticle interference is usually to decrease the scattered intensity at the smallest angles and to have little or no effect on the outer part of the curve. Thus, if one uses the Guinier approximation to determine the radius of gyration of the scattering particle, the effect of the interparticle interference will be to give an apparent radius of gyration which is smaller than the true value. In practice we determine the radius of gyration from the average slope over a finite angular range of the log I *versus* h^2 curve. If we assume that the average slope we measure is equal to the actual slope at the angle h_0 in the middle of the range, then we can define an apparent radius of gyration, R_a, as follows

$$R_a^2 = -3 \left| \frac{d[\log I(h)]}{d(h^2)} \right|_{h=h_0} \quad (10)$$

R_a will in general be a function of both concentration and angle. We can show from eq. 8 that for low concentrations, *i.e.*

$$v_1(2\pi)^{-3/2} >> \epsilon\beta(0)$$

we have

$$R_a(c,h_0) = R_a(0,h_0) - \left[\frac{3(2\pi)^{3/2}N_0}{4R_a(0,h_0)M}\right] \cdot \left[\frac{F_1'(h)\epsilon\beta(h) + F_1(h)\epsilon\beta'(h)}{h}\right]_{h=h_0} c \quad (11)$$

Thus the apparent radius of gyration is a linear function of the concentration at low concentrations regardless of the angle at which we determine it. This is convenient in extrapolating to infinite dilution. If h_0 is large enough, then in some cases $R_a(0, h_0)$, the apparent radius of gyration extrapolated to infinite dilution, may differ appreciably from the true radius of gyration, R. This is because the slope was measured in these cases at an angle outside the Guinier approximation region. We were able to avoid this difficulty with serum albumin since the scattering curve remains Gaussian out to comparatively large angles.

Experimental

An investigation of the angular distribution of the X-rays scattered at small angles by protein solutions requires an

(11) F. Zernicke and J. Prins, *Z. Physik*, **41**, 184 (1927).
(12) H. Mencke, *Physik. Z.*, **33**, 593 (1932).
(13) G. Fournet, *Compt. rend.*, **228**, 1421 (1949a).

intense well-collimated X-ray beam. In this work the X-ray source was a tube with a water-cooled, copper anode about four inches in diameter rotating at 950 r.p.m. The tube was normally operated at 30 kilovolts d.c. and a current of 80 milliamperes. The beam was collimated with a slit system (Fig. 1) used in previous work in this Laboratory.[7,8] For most of the work on serum albumin the slits were each 0.06 cm. wide and 1.0 cm. high. Successive slits were 50 cm. apart. With this arrangement, the total flux incident on the sample was approximately 10^8 photons/sec. and the background scattering with nothing in the scattering position was less than 1 photon/sec. at angles from the central beam greater than 0.005 radian. Monochromatization was achieved with balanced Ross filters of nickel and of cobalt foil which isolate the CuKα line. A Geiger counter was used to detect the X-rays.

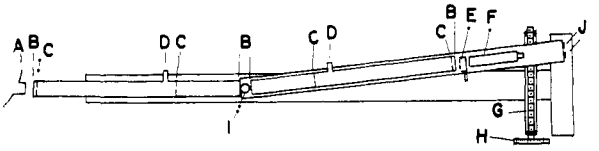

Fig. 1.—Diagram of slit collimating system (scale 10:1): A, X-ray tube exit window; B, mica window; C, tantalum slits; D, vacuum connections; E, Ross filters on slide; F, Geiger counter; G, high precision screw; H, calibrated wheel; I, pivot and sample holder; J, rollers.

The sample holders for the protein solutions were thin-walled glass tubes with a transmission for CuKα X-rays of about 70%. These had the advantage over plastic sample holders of contributing very little to the background scattering. The tubes were made by drawing down Pyrex tubing until the walls were about 0.02 mm. thick and then flattening them by pressing between two pieces of graphite in a furnace until the tubes had a roughly rectangular cross-section of 1 mm. by 3 mm. They were filled by means of a fine pipet and sealed at each end with a drop of paraffin. The sample holders were fastened with Scotch tape to a metal mount which could then be placed in position in the X-ray beam inside a cylindrical cooling shield. The cooling shield was double walled with cold water circulating between the walls to keep the sample at about 10° during a run. This is of importance since the runs usually lasted 8–12 hours.

An adequate correction for background scattering can be made by measuring the scattering from the sample holder filled with solvent and subtracting these readings from the solution scattering. In the region of the Guinier approximation the background scattering was about equal to the protein scattering in a solution with an albumin concentration of 0.25%. Thus the taking of data on solutions more dilute than this was unrewarding. At larger angles the protein scattering dropped off more rapidly than the background so that even with 5% albumin solutions the protein scattering was only one-half the background scattering at 0.05 radian, the largest angle at which data were taken. Figure 2 shows the distribution with angle of the background scattering, and for comparison the scattering from a 1% serum albumin solution. The background scattering is broken down into scattering from solvent, sample holder, the air around the sample holder, and a blank background which is mainly due to scattering from the mica windows of the slit system. The counts due to cosmic rays and local radioactivity are immediately eliminated from all data by the procedure of taking the difference between the readings with a nickel filter and a cobalt filter. The determination of the components of the background requires a more elaborate procedure than is necessary for the routine correction of data. If no background corrections are made, the apparent radius of gyration as determined from a 5% serum albumin solution is 1.5% low. The effect would increase to 8.5% in the case of a 0.5% albumin concentration. However, since the background scattering can be determined to a few per cent., it is a negligible source of error in the radius of gyration.

To determine the effect on the scattering data of the finite size of the collimating slits detailed calculations were made of the smearing effect of slits of various sizes on the theoretical scattering curves for ellipsoids of revolution. A study also was made of the inverse process of correcting the experimental data to eliminate slit effects. A complete report of this work will be given in a later paper. The calculations showed that for a scattering curve with the approximate shape of that of serum albumin and with the slit dimensions given previously, the effects of the finite width and height of the collimating slits on the initial slope of the curve were each considerably less than 1% and of opposite sign. Hence the slit correction to the radius of gyration, which depends on the square root of the initial slope, is negligible.

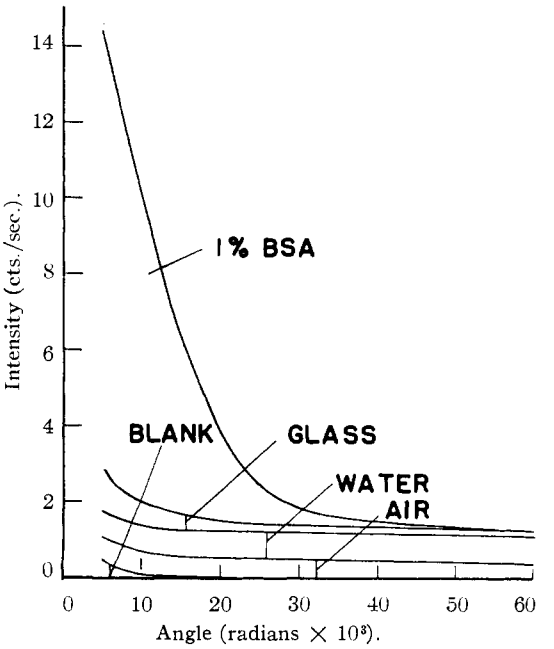

Fig. 2.—Background scattering in comparison to BSA scattering. Each background is added to the one below it.

The scattering curve for serum albumin at larger angles, beyond the region where the Guinier approximation is valid, will be affected to a small extent by slits of the height used in the present work. Corrections were made by the method suggested by Shull and Roess.[14] They have shown that if the experimental scattering curve can be resolved into a sum of Gaussians, $F(\theta) = \Sigma_i T_i\, e^{-t_i^2\theta^2}$, then the slit-corrected scattering curve can be represented by another sum of Gaussians with different term amplitudes, $T_i' = g(t_i)T_i$. The function $g(t_i)$ depends only on the geometry of the scattering apparatus and was calculated in closed form for the slit system in this work. It was found that the serum albumin scattering curve could be fitted quite accurately by a sum of three Gaussians. The results before and after the correction for the effects of the slit heights will be discussed later.

Materials.—The bovine serum albumin used in this work was a crystallized preparation obtained from Armour and Company, Lot 284-8. According to the given specifications, this preparation had a globulin content of less than 0.01%. The human mercaptalbumin monomer and dimer preparations were obtained as frozen solutions of approximately 14% concentration from Professor John T. Edsall of Harvard Medical School. They had been prepared by a further fractionation of human Fraction V following the method of Hughes.[15] Sedimentation studies of both the BSA and HMA preparations showed no evidence of any peaks other than the main one, but a slight asymmetry of the main peak in both cases indicated a few per cent. of material slightly larger than the main component. Since there is such a small amount of this heavy component and since its molecular weight would appear to be only about twice that of serum albumin it should not have a significant effect on the scattering curves.

(14) C. G. Shull and L. C. Roess, *J. Appl. Phys.*, **18**, 295 (1947).
(15) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947).

Most of the samples were prepared by dialyzing against a salt solution of the desired *p*H and ionic strength. The dialyses were carried out for 24 hours at 0° with several changes of the bath. Protein concentrations were obtained by dry weight determinations. For some of the runs on BSA at high concentrations the Armour material was dissolved in distilled water and no dialysis carried out. In these cases the ionic strength was about 10^{-2} and the *p*H about 5.

Results

Monomer.—A series of runs at concentrations varying from 5% down to 0.5% was made on both bovine serum albumin and human mercaptalbumin monomer under essentially isoelectric conditions (*p*H 4.65 in 0.05 *M* NaCl). The runs on BSA are shown in Fig. 3 on a graph of intensity *versus* scattering angle. When plotted on a graph of log intensity *versus* scattering angle squared the runs all gave straight lines (but of different slopes) in the angular region 0.005 to 0.012 radian ($4\pi R\lambda^{-1} \sin \theta/2 =$ 0.6 to 1.5). A typical run plotted in this way is shown in Fig. 4. The extrapolations to zero angle shown in Fig. 3 were made on the basis of the straight line plots.

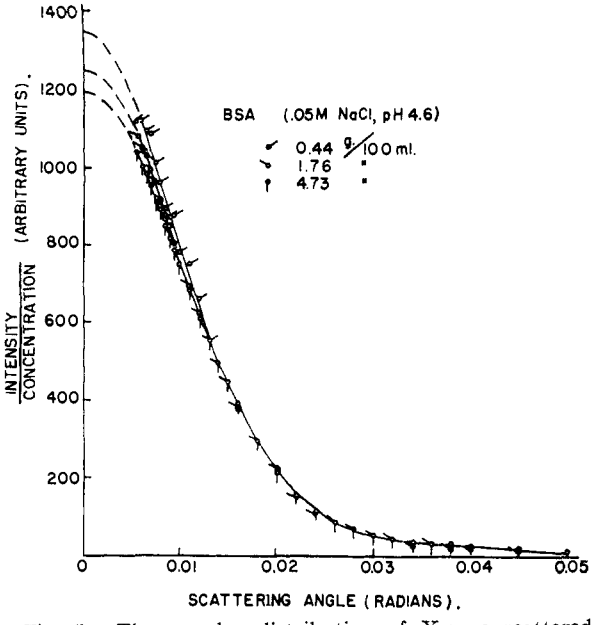

Fig. 3.—The angular distribution of X-rays scattered from solutions of BSA under nearly isoelectric conditions.

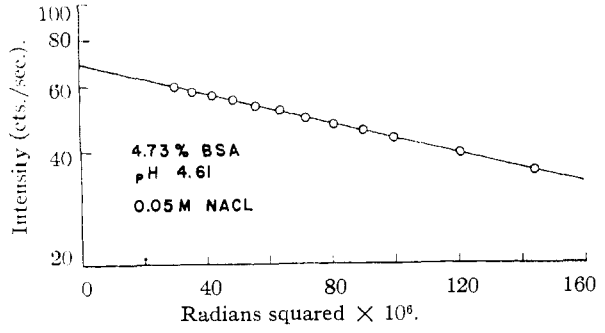

Fig. 4.—The X-ray scattering from a 4.73% BSA solution in the region of the Guinier approximation on a plot of log of scattered intensity *versus* scattering angle squared.

Even at these low concentrations and under approximately isoelectric conditions there was a measurable interparticle interference effect resulting in a steady decrease of the apparent radius of gyration with increasing concentration. In order to eliminate this effect from the final result the apparent radii of gyration were plotted against concentration and the true value found by extrapolation to zero concentration. A straight line extrapolation was found to fit the data within the experimental error in agreement with the theoretical result expressed in eq. 11. The average deviation of the results from this straight line was about 0.5%. The extrapolation to zero concentration is shown in Fig. 5. The extrapolated results for the radii of gyration are 29.8 Å. for bovine serum albumin and 31.0 Å. for human mercaptalbumin monomer.

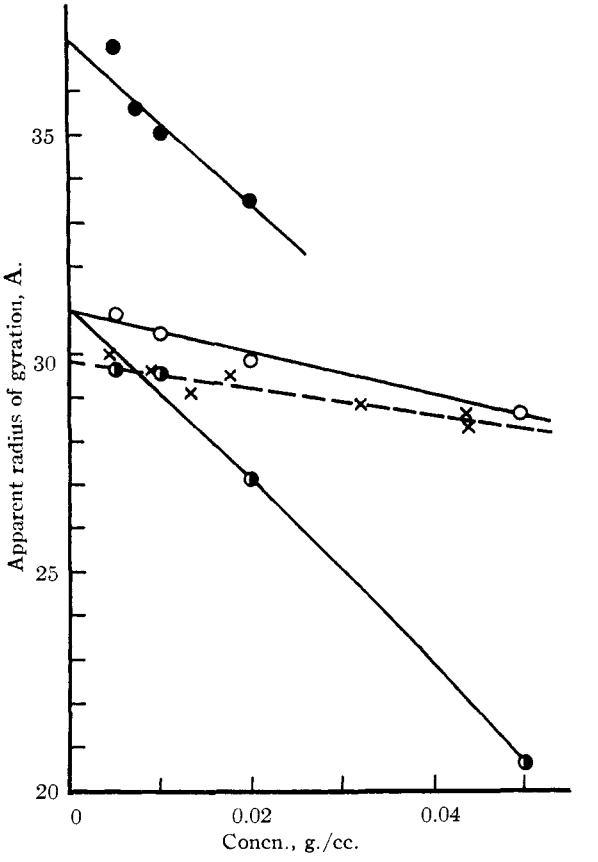

Fig. 5.—Apparent radius of gyration as a function of concentration for solutions of BSA and HMA monomer and dimer: ●, HMA dimer, 0.10 Γ/2 PO_4, *p*H 6.0; ◑, HMA monomer, 0.10 Γ/2 PO_4, *p*H 6.0; ○, HMA monomer, 0.05 *M* NaCl, *p*H 4.7; ×, BSA, 0.05 *M* NaCl, *p*H 4.65.

Since our precision is estimated to be about 1% this difference of 4% in the radii of gyration as determined here is a real difference. Whether it represents an actual difference between the BSA molecule and the HMA molecule in solution or whether it represents instead a difference in purity of the preparations or perhaps an effect due to aggregation or denaturation is a more difficult question. As stated previously, sedimentation studies

on both preparations gave evidence for only a very small amount of a slightly heavier impurity. Even 5% of an impurity of twice the molecular weight of serum albumin present in one solution but not in the other would result in a difference in the apparent radii of gyration of only about 2%. On the other hand it would take no more than 0.5% of an impurity with ten times the molecular weight of serum albumin to account for the entire difference of 4%. Impurities of much larger molecular weights would rapidly become unimportant because their scattering would be confined to angles too small to be observed. It should be noted that the reported values for the molecular weight and sedimentation constant of bovine and human serum albumin are the same within an experimental error of 1 or 2%. These results refer to unfractionated human serum albumin while our X-ray results on human albumin refer to the mercaptalbumin fraction.

Another series of runs was made on the mercaptalbumin monomer at concentrations from 5% down to 0.5% and a *p*H 6.0 in a phosphate buffer of ionic strength 0.10. Under these conditions the interparticle interference effect was considerably larger than under isoelectric conditions, resulting in a much greater decrease with concentration of the apparent radius of gyration. The extrapolated value for the radius of gyration, however, seemed to be the same within the experimental error as obtained under isoelectric conditions. The results of this series of runs are also plotted in Fig. 5.

The results shown in Fig. 5 point out the fact that interparticle interference effects are important even at concentrations of less than 5%. Previous investigators in this field have usually considered it sufficient to keep concentrations below about 10%. It is evident that even under approximately isoelectric conditions this can result in an appreciable error in the radius of gyration and under other than isoelectric conditions it can result in very large errors. This effect undoubtedly explains the difference between the present result of 29.8 Å. for the radius of gyration of bovine serum albumin and the result of 26.6 Å. given in a previous paper by Ritland, Kaesberg and Beeman.[5] It could also explain the even lower result of 23 Å. obtained by Dervichian, Fournet and Guinier[4] on horse serum albumin although there is also the possibility of a species difference. In light scattering experiments these interference effects are even more apparent since the effects increase with decreasing scattering angle and light scattering measurements, due to the longer wave length, are equivalent to X-ray experiments at much smaller angles than those here measured.

The extrapolated results given above for the radii of gyration are the most unambiguous information that can be obtained from the small-angle scattering data. Additional but more ambiguous information on the shape of the molecules can be obtained by comparing the extended scattering curve with the theoretical scattering curves for particles of various shapes. If one assumes for convenience an ellipsoidal model as is commonly done, then one can hope to obtain the axial ratio of the proposed ellipsoid by comparing the complete scattering curve with the theoretically calculated scattering functions for ellipsoids of revolution.

For this purpose extended runs were made on bovine and human albumin at concentrations of 5% and at isoelectric conditions. These runs extended to an angle of 0.05 radians ($4\pi R\lambda^{-1} \sin \theta/2 \simeq 6$) where the intensity was down by almost a factor of one hundred from the central scattered intensity. To eliminate the interparticle interference effects at small angles these runs were combined with runs taken at small angles on more dilute solutions to give composite curves which extended to large angles and were free of interference effects. There was no appreciable difference between the curves for bovine serum albumin and human mercaptalbumin after the abscissa has been adjusted to allow for the 4% difference in radius of gyration, indicating that the two molecules have essentially the same shape.

Before comparing with the theoretical curves, the experimental scattering curve for BSA was corrected for slit effects which became noticeable at angles beyond the region of the Guinier approximation. To make this correction the experimental curve, $F(\theta)$, was resolved graphically into a sum of three Gaussians as

$$F(\theta) = 811e^{-(7.36)^2\theta^2} + 135.5e^{-(56.6)^2\theta^2} + 53.5e^{-(25.5)^2\theta^2} \quad (12)$$

The intensity has been normalized to 1000 at the origin. The slit corrections were then made by calculating the change in the term amplitudes. This gave the following result for the slit-corrected,

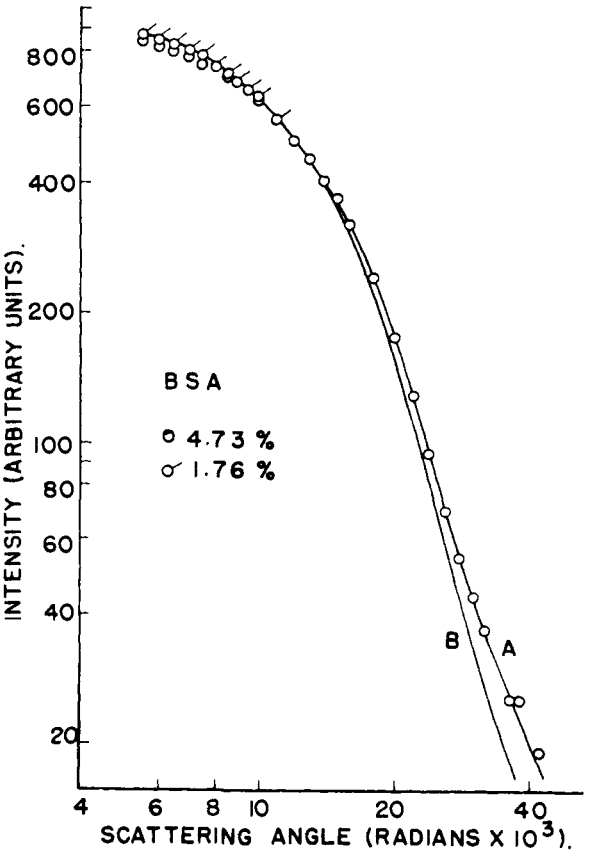

Fig. 6.—The bovine serum albumin scattering curve (A) before slit correction and (B) after slit correction.

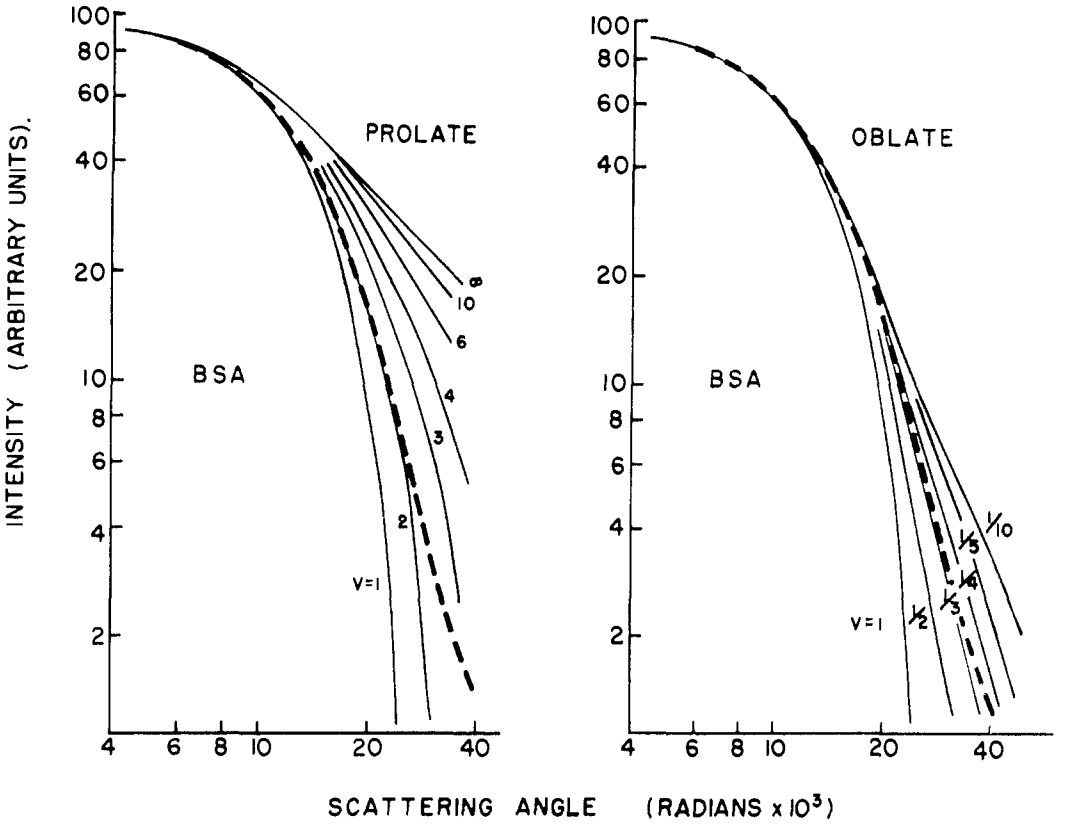

Fig. 7.—A comparison of the slit-corrected scattering curve for bovine serum albumin with the theoretical scattering curves for prolate and oblate ellipsoids of revolution.

BSA scattering curve, $f(\theta)$, after renormalizing to a central intensity of 1000

$$f(\theta) = 845e^{-(7.36)^2\theta^2} + 120e^{-(56.6)^2\theta^2} + 35.4e^{-(25.5)^2\theta^2} \quad (13)$$

In Fig. 6 there is a graph of equation 12 with the experimental points superimposed and below it a graph of equation 13.

Figure 7 shows the comparison of the slit-corrected scattering curve for BSA with the theoretical scattering curves for ellipsoids of revolution, both prolate and oblate. The solid lines are the theoretical curves; the dashed line is the slit-corrected experimental curve (eq. 13). The theoretical curves are all drawn for a radius of gyration of 29.8 Å. It is clear that the experimental curve agrees better with an oblate ellipsoid model than with a prolate model. The experimental curve for serum albumin is in fairly close agreement over the entire intensity range of nearly 100 to 1 with the theoretical curve for an oblate ellipsoid of axial ratio about $(3.5)^{-1}$. A characteristic feature of the experimental curve is a slight positive curvature beyond 0.025 radians ($4\pi R\lambda^{-1} \sin \theta/2 = 3$). This is present on the theoretical curves for oblate ellipsoids but is absent on the corresponding curves for prolate ellipsoids.

Dimer.—A series of runs was also made on solutions of a mercury dimer of human mercaptalbumin. Under proper conditions the dimer is almost free of monomer contamination. A solution at *p*H 6.0 in a sodium phosphate buffer with an ionic strength of 0.10 was obtained from Professor Edsall. This preparation was run at concentrations varying from 5% down to 0.25%. Sedimentation studies showed that the amount of monomer remaining in the solution was less than 10% at a total protein concentration of 2% and remained constant at about 14% at protein concentrations from 1% down to 0.25%. The dimer scattering was corrected for this admixture of monomer by multiplying the monomer scattering curve by the proper factor to take into account the monomer concentration and then subtracting this curve from the observed curve for the dimer preparation. The apparent radii of gyration as obtained from these corrected curves were plotted against concentration and extrapolated to zero concentration as shown in Fig. 5. The extrapolated result for the radius of gyration of the dimer was 37.2 Å.

It can readily be shown that the radius of gyration, R_2, about the center of charge of a rigid system composed of two identical particles each with a center of inversion is given by

$$R_2^2 = R_1^2 + (d/2)^2 \quad (14)$$

where R_1 is the radius of gyration about its own center of charge of each of the individual particles and d is the separation of the centers of charge. Using this relation and the results of 31.0 and 37.2 Å. for the radii of gyration of the HMA monomer and dimer, the separation of the centers of the two components is calculated to be 41 Å.

Interference Effects.—The results discussed so far were derived from the scattering curves after interparticle interference effects had been elimi-

nated by extrapolation to infinite dilution. If instead we concern ourselves with the interparticle interference effects themselves, we can obtain information about the molecular interactions in solution. It is not the purpose of this paper to discuss these effects in detail. We will however give some results for the purpose of indicating that the interference effects are of the magnitude to be expected, thus providing additional evidence for the correctness of the extrapolations referred to above.

From eq. 18 it follows directly that

$$\frac{Kc}{I(h)} = \frac{1}{P(h)}\left[1 - F_1(h)\epsilon\beta(h)(2\pi)^{3/2}\frac{N_0}{M}c \ldots\ldots\right] \quad (15)$$

where K is a constant. Further terms in the series involve higher powers of the concentration, c. Thus if at any value h_0 corresponding to a scattering angle θ_0 ($h = 4\pi\lambda^{-1}\sin\theta/2$) we plot the ratio of the concentration to the scattered intensity *versus* the concentration we should obtain a straight line at small concentrations. The intercept of this straight line with the zero concentration axis determines $P(h_0)$ and the slope of the line determines $F_1(h_0)\epsilon\beta(h_0)$. Doing this at several angles we can determine $\beta(h)$ if we know $F_1(h)\epsilon$, and from $\beta(h)$ we can in theory determine the interparticle potential, $\Phi(r)$, by a Fourier inversion of eq. 9. Alternatively we could determine the radial distribution function $W(r)$ from a Fourier inversion of the scattering curve using eq. 7. To do either of these inversions is beyond the scope of this paper and moreover would be hindered by the fact that both of them require a knowledge of $F_1(h)$, which cannot be accurately calculated until the shape of serum albumin is definitely established. We will, however, attempt to determine $\beta(0)$ since this value of β has special significance and since its determination is simplified because $F_1(0) = 1$. $\beta(0)$ is related to B, the interaction constant which appears in the osmotic pressure equation

$$\frac{P}{RTC} = \frac{1}{M} + Bc \quad (16)$$

by the following relationship

$$\beta(0) = -2M^2B(2\pi)^{-3/2}(N_0)^{-1} \quad (17)$$

Thus for the limiting case of very small angles eq. 16 reduces to

$$\frac{K'c}{I(0)} = \frac{1}{M} + 2Bc \quad (18)$$

which is analogous to the expression for the turbidity which is commonly used in light scattering work.

Using values of $I(0)$ obtained by extrapolating the intensity curves back to zero scattering angle we plotted $c/I(0)$ *versus* c for each of the three sets of data—BSA in 0.05 M NaCl at pH 4.65; HMA in 0.05 M NaCl at pH 4.65; and HMA in 0.10 ionic strength phosphate buffer at pH 6.0. There was a scatter of points due both to uncertainties of extrapolation and also to fluctuations in the intensity of the incident X-ray beam over the period of time required to take the three sets of data. Drawing the best possible straight line, however, and using eq. 18 we obtained the results in the fourth column of Table I.

TABLE I

Protein	$\Gamma/2$	pH	$B \times 10^5$ From eq. 18	$B \times 10^5$ From eq. 19
BSA	0.05 NaCl	4.65	3.8	2.8
HMA	.05 NaCl	4.65	7.4	3.7
HMA	.10 PO_4	6.0	12.3	9.2

We have attempted to compare these results with the values of B determined by Edsall, *et al.*,[16] in their light scattering work on BSA. This comparison cannot be made accurately due to uncertainties in titration data and due to the fact they took their data at other ionic strengths and, in some cases, with other buffers. Making rough interpolations on the graphs of their results, however, we find that they obtained a B of about 2×10^5 in 0.05 M NaCl near pH 4.6 and a B of about 10×10^5 in 0.10 M NaCl near pH 6, in satisfactory agreement with our results.

We would also like to check if the variation of apparent radius of gyration with concentration is what would be expected from the theory of interparticle interference effects. The slopes of the straight lines in Fig. 5 showing the variation of R_a with concentration are given by eq. 11. We see that these slopes depend on the functions $F_1(h)$ and $\beta(h)$ and their first derivatives. Neither of these functions is known for an actual serum albumin solution, but if we assume for the purpose of an order of magnitude calculation that the serum albumin molecules behave in solution like a collection of hard spheres, then we can determine the diameter, D, of these hypothetical spheres from the slope of the lines of R_a *versus* c. Then knowing the relationship between D and the interaction constant, B, we can get another rough estimate of B to compare with that made above. For the case of a collection of hard spheres eq. 11 reduces to

$$R_a(c,h_0) = R_a(0,h_0) - \left[\frac{1.89D^5}{MR_a(0,h_0)}\right]\cdot\left[-\frac{\Phi'(h_0D)}{h_0D}\right] \times c \quad (19)$$

where $\Phi(x)$ is the same function defined in eq. 3. Using eq. 19 and the data in Fig. 5 we obtained the estimates of B shown in column 5 of Table I. We see that the agreement with the estimates made previously on the basis of the extrapolated zero-angle scattering is satisfactory considering the assumptions made. It appears therefore that the observed variation with concentration of both the extrapolated zero-angle scattering and the apparent radius of gyration are in agreement with the theory of interparticle interference effects and with the values of the interaction constant, B, previously measured by light scattering.

Finally we would like to show some data taken at much higher concentrations. Figure 8 shows scattering curves from BSA solutions at concentrations of 5, 21 and 48% (w./v.). The curves show clearly the effect of increasing interparticle interference and the curve for the highest concentration shows an interparticle interference maximum at an angle of 0.019 radian. Considering the diffuseness of the peak this agrees satisfactorily with the peak position of 0.022 radian obtained for this concen-

(16) J. T. Edsall, H. Edelhoch, R. Lontie and P. Morrison, THIS JOURNAL, **72**, 4641 (1950).

tration by Riley and Oster[17] in their work on small-angle X-ray scattering from concentrated solutions of BSA.

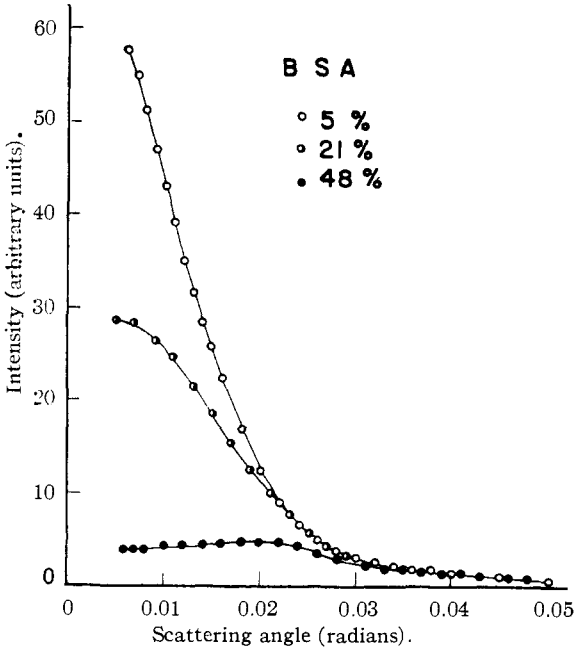

Fig. 8.—Scattering curves for concentrated solutions of bovine serum albumin.

Discussion of Results

We will now discuss the relation of the X-ray measurements to other physical-chemical data on serum albumin. As mentioned previously, this comparison can give information on the hydration of the serum albumin molecule in solution. We will first consider a simple ellipsoidal model and then concern ourselves with the more complex models which seem to be required to fit single crystal X-ray diffraction results.

Ellipsoidal Model.—The single crystal work of Low[18] on human serum albumin gives a molecular weight of 65,600. A recent determination by Gutfreund[19] of the molecular weight of bovine serum albumin from osmotic pressure measurements gives a result of 66,000. Using a value for the sedimentation constant of BSA of $S_{20,w} = 4.30$ Svedberg units,[20-24] a value for the diffusion constant of $D_{20,w} = 5.93 \times 10^{-17}$ cm.^{2}/sec.,[25] and a value for the partial specific volume of 0.734 cm.^{3}/g.[26] we calculate a molecular weight of 66,200 and a frictional ratio of 1.34. In the following discussion then, we will use 66,000 for the molecular weight of both human and bovine serum albumin.

If we assume that the partial specific volume is approximately equal to the reciprocal of the density of the molecule in solution, the above results give a molecular volume of 80,000 Å.^{3}. A knowledge of the volume and the radius of gyration of an ellipsoid of revolution determines the two possible axial ratios, one prolate and one oblate. Hence by assuming an ellipsoidal model and ignoring for a moment the evidence of the extended X-ray scattering curve we determine from the radius of gyration of 29.8 Å. and the molecular volume of 80,000 Å.^{3} an axial ratio for BSA of either 3.5 prolate or $(5.4)^{-1}$ oblate. This approach is analogous to that used in light-scattering work in determining a molecular shape from the molecular weight and dissymmetry of scattering. The axial ratio of BSA was also determined in this way in earlier work from this Laboratory. We see, however, that we now encounter difficulties with this approach since both the axial ratios obtained in this way are in poor agreement with our results for the extended scattering curve as shown in Fig. 7. The disagreement could be due to failure of the assumption that the molecular density in solution is the reciprocal of the partial specific volume. This assumption would be in error if the serum albumin molecule in solution were internally hydrated.

If we discard, therefore, the axial ratios of 3.5 prolate or $(5.4)^{-1}$ oblate calculated above from the radius of gyration and anhydrous molecular volume and use instead the axial ratio of $(3.5)^{-1}$ oblate which is in best agreement with the extended scattering curve, then we can calculate a molecular volume from the X-ray scattering data alone. This volume turns out to be 120,000 Å.^{3} as compared to 80,000 Å.^{3} obtained above for the volume of the anhydrous molecule. The difference could be explained if we assign to the serum albumin molecule in solution an internal hydration of 40,000 Å.^{3} of water. It would have to be an internal hydration, which expands the region of higher electron density, since water bound to the outside of the protein molecule would not affect the X-ray scattering unless the hydrated layer were considerably compressed. An internal hydration of 40,000 Å.^{3} amounts to 0.37 g. of water per gram of protein if we assume that the partial specific volume of the water of hydration is unity.

It is generally assumed that the water bound to proteins in solution is bound to the outside of the molecule, although recent work[7] in this Laboratory has indicated quite conclusively that there is internal hydration in some large nucleoproteins. The present evidence for the internal hydration of serum albumin is much less conclusive. It is quite possible that some model other than an ellipsoid of revolution would give a scattering curve in agreement with our results and not require the assumption of internal hydration. One such model will be discussed below, but in that case too there seems to be some preference for a volume larger than the anhydrous volume of 80,000 Å.^{3}.

Additional information on the hydration may be obtained from the frictional ratio. Using the value of 1.34 for the frictional ratio of BSA calculated above from the sedimentation and diffusion constants and partial specific volume and using an axia

(17) D. P. Riley and G. Oster, *Discs. Faraday Soc.*, **11**, 107 (1951).
(18) B. Low, THIS JOURNAL, **74**, 4830 (1952).
(19) H. Gutfreund, *Trans. Faraday Soc.*, **50**, 628 (1954).
(20) G. Kegeles and F. J. Gutter, THIS JOURNAL, **73**, 3770 (1951).
(21) J. F. Taylor, *Arch. Biochem. Biophys.*, **36**, 357 (1952).
(22) G. L. Miller and R. H. Golder, *ibid.*, **36**, 249 (1952).
(23) J. M. Greeth, *Biochem. J. (London)*, **51**, 10 (1952).
(24) S. Shulman, *Arch. Biochem. Biophys.*, **44**, 230 (1953).
(25) D. F. Akeley and L. J. Gosting, THIS JOURNAL, **75**, 5685 (1953).
(26) M. O. Dayhoff, G. E. Perlmann and D. A. MacInnes, *ibid.*, **74**, 2515 (1952).

ratio of $(3.5)^{-1}$, Oncley's contour diagram[27] gives a total hydration of 0.48 g. of water per g. of protein. Assuming an internal hydration of 0.37 this indicates an additional 0.11 g. of water per g. of protein bound to the outside of the albumin molecule. The uncertainties in these hydrations due to the uncertainties in the measured quantities are difficult to estimate but they are quite large. The additional uncertainties due to the assumptions about ellipsoidal shape, partial specific volume of the water of hydration, etc., are even more difficult to estimate.

Viscosity measurements on solutions of human serum albumin reported by Oncley, Scatchard and Brown[28] give an intrinsic viscosity of 0.042 or a viscosity increment of 5.7. From another contour diagram of Oncley,[27] the total hydration of a molecule can be determined if the viscosity and axial ratio are known. A viscosity increment of 5.7 and an axial ratio of $(3.5)^{-1}$ gives a total hydration of 0.40 g. of water per g. of protein which is in reasonable agreement with the result of 0.48 obtained above.

Thus if we assume that the bovine serum albumin molecule is an ellipsoid of revolution we find an axial ratio of $(3.5)^{-1}$, a radius of gyration of 29.8 Å., an internal hydration of 0.37 g. of water per g. of protein, and an external hydration of 0.11 to be consistent with our X-ray scattering results and the results of sedimentation, diffusion and viscosity measurements. The picture is essentially the same for the human mercaptalbumin molecule except for the slightly greater radius of gyration of 31.0 Å.

Other Models.—The simple ellipsoidal model we present above is not in agreement with that suggested by Oncley[28] on the basis of dielectric dispersion measurements and other data. He suggested as a model for the human serum albumin molecule a prolate ellipsoid 150 Å. long and 38 Å. in diameter with a hydration of 0.2 g. of water per g. of protein. From viscosity and sedimentation studies on human mercaptalbumin Oncley[29] also suggested an alternative model described as a modified right prism 150 Å. long, 36 Å. wide, and 38 Å. high. In a recent X-ray study of crystalline human serum albumin Low[18] shows that a molecular model similar to Oncley's modified prism packs well into the unit cell of human mercaptalbumin dimer and of human decanol albumin. A dimer molecule composed of two oblate ellipsoids of revolution each with a radius of gyration of 31 Å. and an axial ratio of $(3.5)^{-1}$ cannot pack into the mercaptalbumin dimer unit cell measured by Low. Ellipsoids with the above radius of gyration and axial ratio would be 27.5 Å. high and have a diameter of 96 Å. They would have to be oriented with their axis of revolution parallel to the *b* axis of the crystal but since the *c* dimension of the unit cell is only 51.5 Å. in the "air-dried" crystal and 63 Å. in the "wet" crystal it appears that the simple ellipsoidal model cannot be made consistent with the X-ray diffraction data. It is possible there are some shape changes as the monomer goes into the dimer but these would have to be considerable in order to be of help and besides it appears to be equally difficult to pack the above ellipsoids into the human serum decanol albumin unit cell described by Low.

On the other hand, right prisms of the dimensions suggested by Oncley[29] and Low[18] are not in agreement with our small-angle scattering results. Even the shortest model (130 × 22 × 50 Å.) suggested by Low for the mercaptalbumin molecule has a radius of gyration of 33.2 Å., significantly higher than our result of 31.0 Å., and a roughly prolate shape in disagreement with our extended scattering curve. In addition, the type of packing proposed by Low for the mercaptalbumin dimer crystals requires that the center to center separation of the two monomer units in the dimer be at least half the *a* dimension of the unit cell, that is 74 Å. in the "air-dried" crystal and 82.5 Å. in the "wet" crystal. This is twice as great as the 41 Å. separation calculated from the measured radii of gyration of monomer and dimer.

In attempting to find a model consistent with all of the X-ray data we have been led to consider a rectangular parallelelpiped. Such a model with dimensions of 74 × 22 × 49 Å. gives the correct anhydrous volume of about 80,000 Å.^{3} ($0.734(N)^{-1}$ × 65,600) and a dimer of two such molecules can be packed into the unit cell of the "air-dried" mercaptalbumin dimer crystal and satisfy the space group requirements. Moreover the amount of overlap of the two monomer units in the dimer is now entirely at one's disposal and a center to center separation of 41 Å. can be achieved. The strong (040) reflection in the dry crystal could be explained by assuming a double layer structure for the monomer. This model, however, gives a radius of gyration of only 26.4 Å. Thus to obtain the monomer radius of gyration of 31.0 Å. we must again assume that the molecule in solution is swelled by internal hydration. Figure 9a shows the packing into the unit cell of the "wet" dimer crystal of a parallelepiped with its dimensions increased to 82.5 × 27.5 × 63 Å. which give a radius of gyration of 31.0 Å. This model has a volume of 143,000 Å.^{3} corresponding to an internal hydration of 0.59 g. of water per g. of protein. In addition to the fact that such a large internal hydration seems unlikely it appears that this model also may be too symmetric to be in good agreement with our extended scattering curve.

Somewhat more promising results are possible if we drop the necessity of a 41 Å. center to center separation of the two monomer units of the dimer in the "air-dried" dimer crystal. This might still be consistent with our measured separation of 41 Å. in solution if, for instance, the two molecules of the dimer in solution were able to rotate about the –S–Hg–S– bond either freely or to a new position different from that taken in the dry crystal. With this assumption we may consider a right prism which packs into the crystal structure as suggested by Low but which has modified dimensions so as to give a radius of gyration of 31 Å. A prism 106 × 21.5 × 50 Å. has a volume of 79,000 Å.^{3}, a radius of gyration of 31.0 Å. and packs into the mercaptal-

(27) J. L. Oncley, *Ann. N. Y. Acad. Sci.*, **41**, 121 (1941).

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

(29) J. L. Oncley, personal communication quoted by B. Low.[17]

bumin dimer crystal as shown in Fig. 9b. It is not obvious without a detailed calculation to what extent this model would agree with our extended scattering curve. Qualitatively, however, it appears that the model may be somewhat too asymmetric and have too small a volume to agree well with the observed scattering at larger angles.

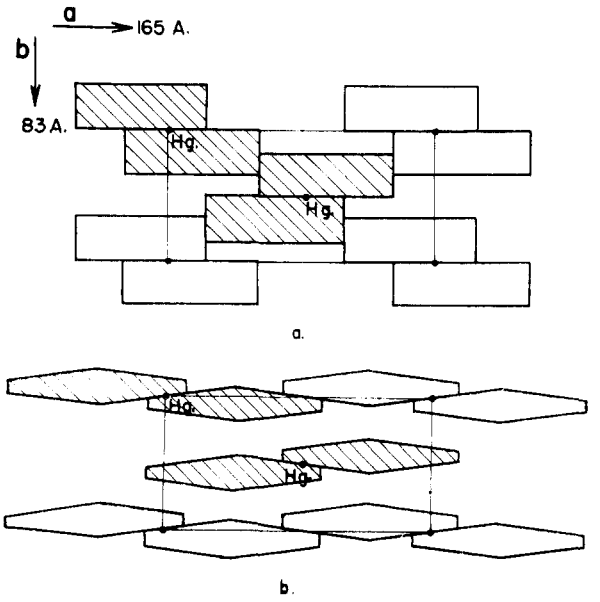

Fig. 9.—Packing of two proposed models for serum albumin molecule into the mercaptalbumin dimer "wet" unit cell: (a) 8.25 Å. rectangular parallelepiped, (b) 106 Å. right prism.

To review briefly, the 82.5 Å. rectangular parallelepiped and 106 Å. right prism described above both give a radius of gyration of 31 Å. and both pack into the crystal structure described by Low. The parallelepiped has a volume much bigger than the dry volume of HMA and hence necessitates the assumption of a large internal hydration of 0.59 g. of water per g. of protein; the prism has a volume just equal to the dry volume of HMA and thus requires no internal hydration. The parallelepiped appears to be too symmetric and the prism too asymmetric to be in good agreement with the scattering data at larger angles, which agrees well with the scattering curve for an oblate ellipsoid with axial ratio of $(3.5)^{-1}$. These conclusions have not been checked, however, by a calculation of the scattering function for either the parallelepiped or the prism. Figure 10 shows a cross-sectional view and a top view of the 82.5 Å. parallelepiped, the 106 Å. prism, and the 96 Å. oblate ellipsoid of revolution. It will be noted that there is some similarity among the three models. A shorter but wider prism could still give the correct dry volume and crystal packing and with some internal hydration in solution could also give a radius of gyration of 31 Å. Only the parallelepiped, however, of the models we have considered, could give a center to center separation for the dimer in the "air-dried" crystal of 41 Å. Calculation of the scattering functions for these and other models so that their agreement with the extended scattering curve could be checked should help to establish a satisfactory model for the serum albumin molecule.

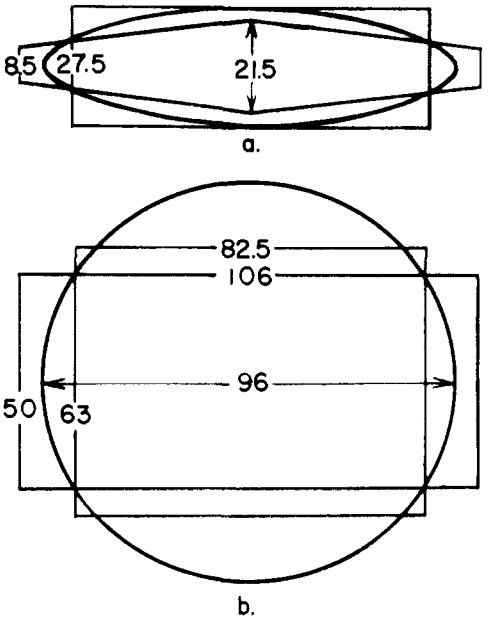

Fig. 10.—Comparison of various proposed models for serum albumin molecule: (a) side view, (b) top view.

Model	Dimension, Å.	R, Å.	Vol., Å.^{3} $\times 10^{-3}$
Right prism	106 × 21.5 × 50	31.0	79
Ellipsoid of revolution	96 × 27.4 × 96	31.0	132
Rectangular parallelepiped	82 × 27.5 × 63	31.0	143

In the above discussion we have attempted to interpret our X-ray results in terms of molecular models with uniform electron density. This is certainly valid if the only density fluctuation in the protein molecules are on an atomic scale since such small spacings produce negligible phase differences at the angles involved here. We have made a simple calculation to determine if such an interpretation is still valid with the longer wave length density fluctuations to be expected in molecules made up of a close-packed collection of coiled polypeptide chains. Since the scattering in the region of the Guinier approximation depends only on the radius of gyration, it was necessary only to calculate the effect of a given intramolecular structure on the radius of gyration to find its effect on the scattering in that region.

For ease in calculation we assumed the serum albumin molecule to be a box with dimensions 80 × 60 × 33 Å. This can be approximated by three rows of close-packed cylinders 12 Å. in diameter and 80 Å. long with 5 cylinders in each row. Each cylinder represents a coiled polypeptide chain. Since the density will be higher along the backbone of the chain than in the side-chain region, we assumed all the electrons to be concentrated in rods 5.5 Å. in diameter along the axis of each cylinder. This corresponds roughly to the diameter of the Pauling and Corey α-helix.[30] We found the radius

(30) L. Pauling and R. B. Corey, *Proc. Nat. Acad. Sci. U. S.*, **37**, 205 (1951).

of gyration of this collection of rods to be less than 1% smaller than the radius of gyration of the box if it were uniformly filled. Even if we let the diameter of the central rods go to zero as their density became infinite the difference is still less than 1%. Also changing the 12 Å. center to center separation of the chains over the range from 10 to 15 Å. has little effect on the results. While the assumed intramolecular structure is an obvious oversimplification, the calculation does indicate that in the region of the Guinier approximation density fluctuations due to the polypeptide chains in the protein molecule will not affect the scattering. An interpretation of this part of the scattering curve in terms of models with uniform density should give valid results for the over-all dimensions of the molecule. A closer study would be necessary to determine at what angles the intramolecular structure becomes important in the interpretation of the scattering data.

Acknowledgment.—We wish to thank Professor John Edsall of Harvard University for generously giving us the human mercaptalbumin monomer and dimer preparations.

MADISON, WISCONSIN

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF SOUTHERN CALIFORNIA]

A Re-evaluation of the Spectral Change Method of Determining Critical Micelle Concentration[1]

BY PASUPATI MUKERJEE[2] AND KAROL J. MYSELS

RECEIVED NOVEMBER 16, 1954

The critical micelle concentration (CMC) is most frequently determined these days by spectral change methods involving color changes of dyes, particularly of pinacyanol, as the detergent concentration changes. A critical examination of this method as applied especially to sodium lauryl sulfate (NaLS) is presented. It is shown that the color change involves the formation of a dye–detergent salt which is highly insoluble but forms a coarse and quite stable suspension in the presence of somewhat more than stoichiometric amounts of detergent. For pinacyanol this salt is red. The presence of the insoluble salt induces the formation of mixed blue micelles at concentrations substantially below the CMC of NaLS itself. The complete solubilization of the dye–detergent salt and therefore the apparent CMC determined by this method depends on a number of factors such as the concentration of dye, the absolute value of the CMC and the temperature. As a result large errors in both absolute and relative values of CMC's are being introduced by the use of this method. The composition of induced mixed micelles and their transition to normal micelles are briefly discussed.

At concentrations generally used (10^{-5} to 10^{-4} M) the dye pinacyanol gives a violet solution in water. In the presence of low concentration of many detergents (*e.g.*, 0.03–0.16% sodium lauryl sulfate) it gives a red color. At only slightly higher concentrations (0.22% and up NaLS) a blue solution is produced. The color change between red and blue occurs not very far from the critical micelle concentration (CMC) of the detergent. The same type of behavior is shown by a number of other dyes. The concentration at which the color change occurs is easy to determine experimentally and ever since Harkins and co-workers[3-6] proposed this "spectral change method" of determining the CMC it has become by far the most popular one. The articles describing its use are too numerous to be all quoted and so only a few are cited.[3-11]

In addition to the determination of a large number of CMC's on a variety of systems several structural interpretations are based on the observed color changes and the effects of various factors on the CMC. We shall discuss some of these later.

There have been indications that the method may not be completely reliable: Klevens noted that the values thus found for the CMC tend to be lower than by other methods.[12] Nor was the agreement between different investigators satisfactory[6,9] nor between visual and colorimetric methods of the same investigator.[13] The principal uncertainty of the method arose probably from the insufficient understanding of the reasons for the observed color changes.

The CMC can be defined unambiguously[14] and determined accurately by three independent methods as shown in Fig. 6 and discussed further below. The pinacyanol method, however, gives a discordant result. This led to the present investigation of the color changes of pinacyanol and to the conclusion that, in practice, the method gives only a rough approximation of the CMC.

Experimental

Materials.—The NaLS used has been described.[14] The pinacyanol chloride was obtained from Eastman Kodak Co. Its microanalyses, either as obtained or after recrystallization, failed to give reproducible results in two laboratories. This seems to be due to incomplete combustion of the chloride salt. The lack of change of absorption coefficient upon recrystallization and the microanalysis of the other salts reported below convince us that the purity of the material was quite satisfactory for our purpose.

(1) Presented in part before the Division of Colloid Chemistry at the New York Meeting of the American Chemical Society, September, 1954.

(2) Colgate–Palmolive–Peet Pre-Doctoral Fellow.

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(6) M. L. Corrin and W. D. Harkins, *ibid.*, **69**, 683 (1947).

(7) H. B. Klevens, *J. Phys. Colloid Chem.*, **54**, 1012 (1950).

(8) (a) S. H. Herzfeld, M. L. Corrin and W. D. Harkins, *ibid.*, **54**, 217 (1950); (b) discussion of reference 21.

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