The Use of Small-Angle X-Ray Scattering to Determine Protein Conformation

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Small-angle X-ray scattering is one of a variety of methods usable for determining structural parameters of proteins in solution. Scattering is unique in its ability to furnish independent information on molecular size and shape, as well as on the thermodynamics of solution behavior. In addition to the radius of gyration of a biological macromolecule, it is possible, with absolute-scale intensity measurements, to obtain the molecular weight, surface-to-volume ratio, hydrated volume, and degree of hydration. A new instrument, designed on the basis of the Guinier and Luzzati approaches, is described. To illustrate use of the technique, results from studies of β-lactoglobulin, α-lactalbumin, lysozyme, and ribonuclease are presented.

Among the methods available for the characterization of globular proteins, small-angle X-ray scattering is one of the most versatile. When it is used on the absolute-intensity scale, this method is capable of yielding the molecular weight, radius of gyration, hydrated volume, surface-to-volume ratio, and degree of hydration of a particle in solution (Beeman *et al.*, 1957; Guinier and Fournet, 1955; Luzzati, 1960; Timasheff, 1963, 1964). In addition to these molecular parameters, one may obtain the thermodynamic parameters of interacting systems (Timasheff, 1963), such as association constants of aggregating subunit systems and the degree of preferential interaction of proteins with components of mixed solvent systems.

With the development in recent years of absolute-intensity apparatus, several proteins have been examined, and their conformational states and degree of association have been characterized. These studies include the acid expansion of bovine serum albumin (Luzzati *et al.*, 1961b), the association equilibrium of β-lactoglobulin (Timasheff and Townend, 1964; Witz *et al.*, 1964), the comparison of α- and δ-chymotrypsins with the zymogen (Krigbaum and Godwin, 1968), and the state of aggregation of glutamate dehydrogenase (Sund *et al.*, 1969).

We have developed a new absolute-intensity instrument and have applied it to the characterization of several proteins. A description of the instrument and its application to the measurement of several radii of gyration have been presented earlier (Pessen *et al.*, 1970). Use of the instrument has now been extended to absolute measurements as well as to a greater number of proteins, to a greater angular range, and thus to the determination of additional parameters. The present paper will describe the results of this work.

THEORETICAL

The fundamental phenomenon of small-angle X-ray scattering is the reemission of electromagnetic radiation with unchanged frequency by the electrons with which it interacts. Since the reemission occurs in all directions, it has the appearance of scattering. For particles which are large relative to the wavelength of the radiation, the interference between radiation reemitted by the individual electrons results in an angular dependence of the scattering intensity characteristic for the geometry of the particle; this effect was first analyzed by Debye (1915). Using the Debye equation, Guinier (1939) showed that, for a point source of radiation, the following asymptotic relation is true, independent of any assumption regarding the general form of the particle

$$i(s) = i(0)\exp(-4/3\pi^2 R_g^2 s^2), \qquad s \equiv 2\sin\theta/\lambda \tag{1}$$

where $i(s)$ is the scattering intensity at an angle measured by s (in the case of a solution, the excess scattering, *i.e.*, the difference in scattering between solution and solvent), $i(0)$ is that same function extrapolated to zero angle, R_g is the radius of gyration (*i.e.*, the root-mean-square of the distances of all the electrons of the particle from its center of electronic mass), θ is one-half the angle between incident beam and the direction in which the scattering is observed, and λ is the wavelength of the incident radiation.

As a practical matter it is rarely possible to utilize a point source because of its insufficient intensity. The geometry generally chosen for the source is one defined by a narrow slit. If the slit is long, so that its height exceeds the angular range, measured at the detector, at which observable scattering occurs, it is said to be an "infinitely high" slit. In analogy to eq 1, the scattering intensity $j(s)$ from an infinite-slit source is given (Guinier and Fournet, 1955; Luzzati, 1960) by

$$j(s) = j(0)\exp(-4/3\pi^2 R_a^2 s^2) + \phi(s) \tag{2}$$

where $j(0)$ is $j(s)$ extrapolated to zero angle, R_a is the apparent radius of gyration, *i.e.*, that referring to a finite concentration of solute, and $\phi(s)$ is a residual function expressing the difference between the gaussian portion of eq 2 and the scattering actually observed; $i(0)$ and $j(0)$ are related by

$$i(0) = 2\sqrt{\pi/3}\, j(0)R_a - 1/\pi \int_0^\infty s^{-2}\phi(s)\mathrm{d}s \tag{3}$$

The theoretical point-source scattering curve can be constructed from the experimental infinite-slit data by an appropriate mathematical transformation (Guinier and Fournet, 1955) which is fairly simple in principle but in practice is attended with considerable difficulties. It is usually carried out numerically on a digital computer.

From the point source data, a number of further parameters may be obtained. The mass of the particle, m, expressed as the number of electrons per particle, is given by

$$m = m_{app} + 2Bm^2 c_e,\ m_{app} \equiv i_n(0)(1 - \rho_0\psi)^{-2}c_e^{-1} \tag{4}$$

Here m_{app}, as defined, is an apparent mass calculated for each finite concentration at which scattering measurements are

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made and is equal to m if the virial effect is negligible, B is the second virial coefficient, which has the same significance as in light scattering and osmometry, c_e is the solute concentration expressed as the ratio of the number of electrons of solute to that of solvent, the subscript n refers to intensities normalized (separately for solution and solvent) with respect to the total scattered energy attributable to the incident beam (*i.e.*, absolute-scale scattering intensity), ρ_0 is the electron density of the solvent, and ψ is the electron partial specific volume of the solute in compatible units. Extrapolation to zero concentration of a plot of m_{app} *vs.* c_e leads, then, to m from the ordinate intercept and, with m known, to B from the slope. The molecular weight, M, is readily obtained from m since

$$M = mN_A/q \tag{5}$$

where q is the number of electrons per gram of the particle calculated from its chemical composition, and N_A is Avogadro's number.

For an isotropic particle of uniform electron density, at large values of s,

$$\lim_{s\to\infty} s^3 j_n(s) = A + \delta^* s^3, \quad A \equiv \lim_{s\to\infty} s^3 j^*_n(s),$$

$$j^*_n(s) \equiv j_n(s) - \delta^* \tag{6}$$

where A and δ^* are constants, $j_n(s)$ is the normalized scattering intensity analogous to $i_n(s)$ above, and $j^*_n(s)$ is a corrected quantity defined by the equation. A plot of $s^3 j_n(s)$ *vs.* s^3 thus yields δ^* and A, and this permits the calculation of several other parameters (Luzzati *et al.*, 1961a).

The external surface area, S, of the particle in solution is given by (Luzzati, 1960; Porod, 1951; Soulé, 1957)

$$S = 16\pi^2 A(\rho_1 - \rho_0)^{-2} \tag{7}$$

where ρ_1 is the mean electron density of the hydrated particle. The hydrated volume, V, can be obtained by integration under the scattering curve

$$V = \frac{i_n(0)}{\int_0^\infty 2\pi s j^*_n(s)\mathrm{d}s} \tag{8}$$

It may be shown that the surface-to-volume ratio is

$$\frac{S}{V} = \frac{8\pi A}{\int_0^\infty s j^*_n(s)\mathrm{d}s} \tag{9}$$

The excess electron density of the hydrated particle over that of solvent, $\Delta\rho = \rho_1 - \rho_0$, can be calculated from

$$\Delta\rho = \frac{\int_0^\infty 2\pi s j^*_n(s)\mathrm{d}s}{c_e(1 - \rho_0\psi)} + \rho_1 c_e(1 - \rho_0\psi) \tag{10}$$

The degree of hydration, H, expressed as the ratio of the number of electrons of water of hydration to the number of electrons of the dry particle, is

$$H = \frac{\rho_0(1 - \rho_1\psi)}{\Delta\rho} \tag{11}$$

The exact values of the parameters of eq 8–11 are obtained by extrapolation to zero protein concentration.

With a knowledge of a number of molecular parameters—namely, M, R_g, V, and S/V—the possible overall geometry of the unknown particle becomes highly restricted. Further information on the particle shape may be obtained from scattering at higher angles. At these angles ($2\Theta > 2°$) the X-ray scattering curves develop maxima and minima superimposed on the Guinier relationship. The positions of these are well defined for different geometric models. Comparison of the experimental curves with those calculated for various likely models then suggests the choice most compatible with the data. Besides furnishing structural parameters, scattering may be used to characterize protein–protein and protein–solvent interactions by means of radial distribution functions, virial coefficients, and the study of geometric changes as a function of environment.

EXPERIMENTAL

Apparatus. The apparatus employed was a new instrument based in part on the original approach of Guinier (1939) as developed by Luzzati *et al.* (1963). Its design principles and special features have been described in some detail elsewhere (Pessen *et al.*, 1970). To summarize briefly, aside from conventional X-ray generating and signal processing circuitry, the instrument consists of two major assemblies: a horizontal X-ray tube and tube housing with attached focusing monochromator, and a horizontal goniometer with attached slits and detector, both mounted on a 2 ft × 4 ft granite surface plate, SP (Figures 1 and 2). (For clarity in showing apparatus details, shielding consisting of lead, lead-impregnated vinyl sheets, and lead-glass windows of appropriate thickness to prevent any radiation hazard, supported by a framework normally surrounding the entire instrument, has been removed.) The X-ray source, X, a fine-focus copper-target diffraction tube, produces a vertical focal line viewed at a 6° take-off angle by the monochromator. The monochromator housing, MC, holds a massive clamp which maintains a thin quartz plate elastically bent to a 1300-mm radius; the faces of the plate are cut at an 8° angle with respect to its $10\bar{1}1$ lattice planes. The bent quartz crystal is capable of isolating the α_1 line from the copper K_α doublet at a Bragg angle of 13° 19.3′. The asymmetry of the lattice planes affords a relatively large distance to be traversed by the converging beam between its exit from the monochromator and the focal plane, where it is observed. Arranged in this optical path are two specially constructed beam-defining slits, S_1 and S_2, and a holder for a liquid-sample cell, SC, all mounted on the goniometer table, G. Rotatable about the central axis of this cell is the scanning arm, SA, carrying a filter holder, F, for accommodating a suitable combination of calibrated nickel-foil filters, used to attenuate the incident beam so that it may be measured on the same scale as the scattered radiation. The scanning arm also carries the receiving slit, S_3, which defines the angular position of observation, an anti-scatter slit, S_4, used to

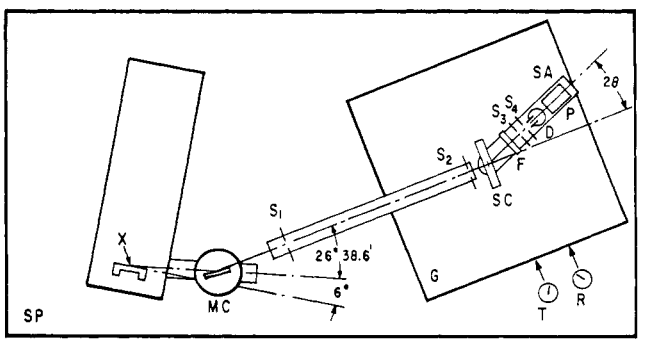

Figure 1. Schematic top view showing layout of X-ray scattering apparatus

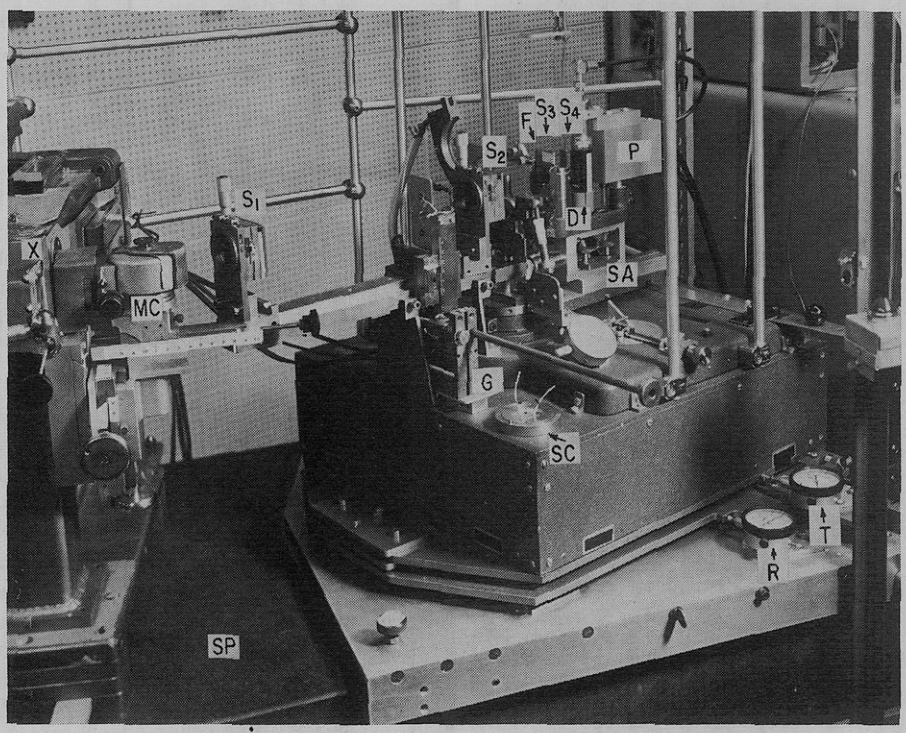

Figure 2. Photographic view showing source, goniometer, and sample cell

eliminate stray radiation, and the detector, D, a sealed-window proportional counter tube with associated preamplifier, P. The goniometer table is the top of a rigid case containing the goniometer drive, change gears, and angular position readout. The case is mounted on the surface plate in such a way as to permit the very precisely adjustable translational and rotational motions (shown by dial indicators, T and R) needed to facilitate the exceedingly critical alignment of the four-slit system with the narrow monochromatized beam. Other fine adjustments for every required degree of freedom of alignment are provided for the monochromator mount, the monochromator crystal, each of the slits, and the cell holder. The main distinction of this apparatus lies in the combination of advantageous features which either are new or have not been incorporated previously in one instrument and which result in mechanical stability and precision, ease of adjustment, and flexibility in use.

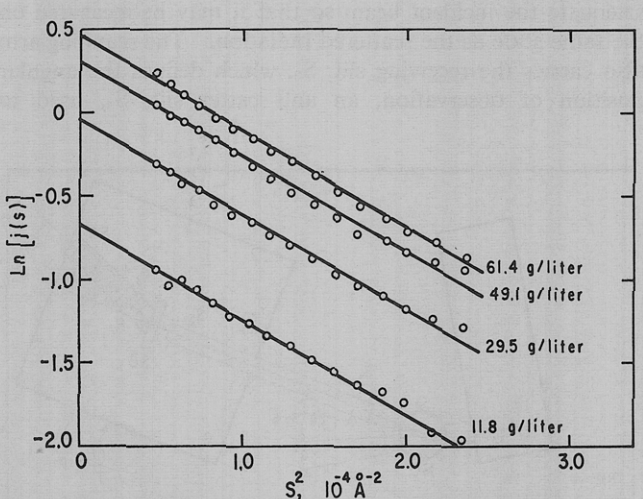

Figure 3. Guinier plots for β-lactoglobulin (intensities not normalized)

Materials and Techniques. The β-lactoglobulin *B* and the α-lactalbumin were prepared from the unpasteurized milk of homozygous *B*/*B* cows and from pooled milk, respectively, according to the procedures of Aschaffenburg and Drewry (1957). Hen's egg white lysozyme was the three-times crystallized material of Pentex, Inc., Kankakee, Ill. Bovine pancreatic ribonuclease was the salt-free five-times crystallized product of Mann Research Laboratories, Inc., Orangeburg, N.Y. Homogeneities of these proteins were validated by sedimentation velocity experiments, which in all cases showed a single gaussian peak. Protein concentrations, ranging roughly from 10 to 50 g/l., were determined spectrophotometrically with absorptivity values of 0.96 l./g-cm for β-lactoglobulin at 278 nm (Townend *et al.*, 1960), 2.01 for α-lactalbumin at 280 nm (Kronman and Andreotti, 1964), 2.60 for lysozyme at 280 nm (Kanarek, 1963), and 0.698 for ribonuclease at 277.5 nm (Eaker, 1962), respectively. Measurements on β-lactoglobulin were carried out in 0.1 *M* acetate buffer at pH 5.7, as in the work of Witz *et al.* (1964); measurements on α-lactalbumin were carried out in 0.1 *M* NaCl at pH 7.0 and those on lysozyme in 0.15 *M* NaCl at pH 3.8, both following Krigbaum and Kügler (1970); and those on ribonuclease were carried out in 0.1 *M* acetate buffer at pH 5.2.

The values of the partial specific volume, $\bar{v}$, used were 0.751 ml/g for β-lactoglobulin (Pedersen, 1936), 0.729 ml/g for α-lactalbumin (Gordon and Ziegler, 1955), 0.7138 ml/g for lysozyme (Charlwood, 1957), and 0.7075 ml/g for ribo-

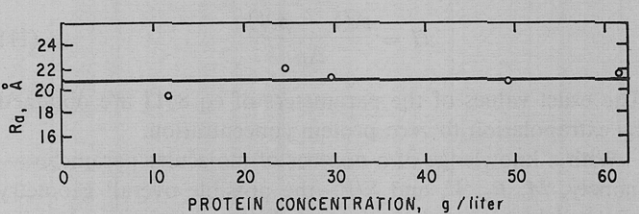

Figure 4. Concentration plot of R_a for β-lactoglobulin

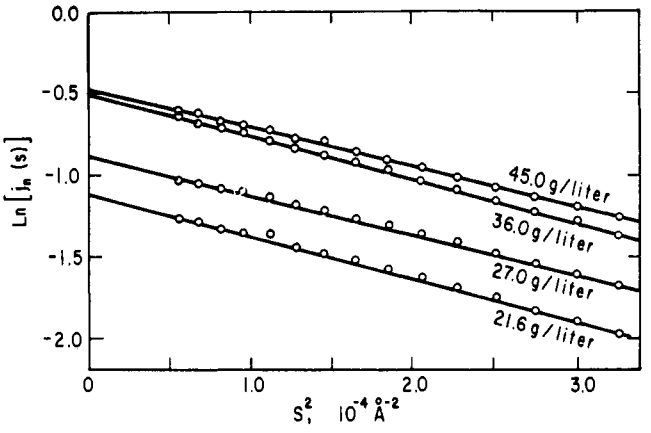

Figure 5. Guinier plots for lysozyme (intensities normalized)

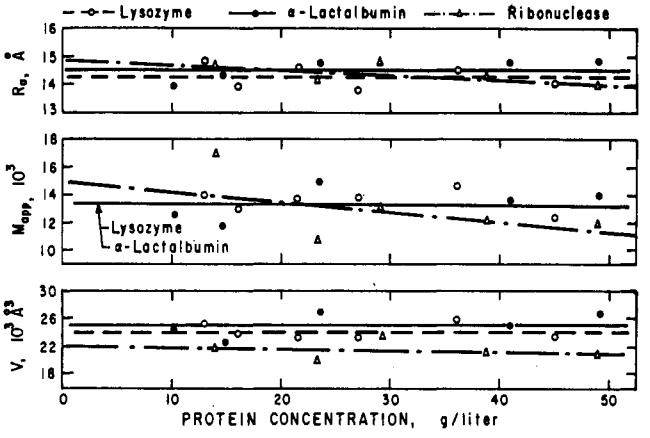

Figure 7. Concentration plots of R_a, M_{app}, and V for three proteins

nuclease (Fahey *et al.*, 1969; Rothen, 1940). The number of electrons per gram, q, of each protein was calculated from the amino acid composition (Gordon *et al.*, 1961; Brew *et al.*, 1967; Canfield, 1963; Smyth *et al.*, 1963, respectively). The resulting values of q and of the electron partial specific volume, $\psi = \bar{v}/q$, were 0.3221 $\times$ 10^{24} electron/g and 2.332 Å^3/electron for β-lactoglobulin; 0.3178 $\times$ 10^{24} electron/g and 2.293 Å^3/electron for α-lactalbumin; 0.3209 $\times$ 10^{24} electron/g and 2.225 Å^3/electron for lysozyme; and 0.3206 $\times$ 10^{24} electron/g and 2.207 Å^3/electron for ribonuclease. A calculated value of ρ_0 = 0.335 electron/Å^3 was used for the solvents.

RESULTS AND DISCUSSION

β-Lactoglobulin. The data for β-lactoglobulin, collected for scattering angles from $-2°$ to $+2°$, were treated according to eq 1 and the resulting Guinier plot is shown in Figure 3. The plot of R_a *vs.* c (Figure 4) gives a value of R_g = 20.8 $\pm$ 0.4 Å. This compares with a value of 21.7 $\pm$ 0.2 Å, reported by Witz *et al.* (1964)

Lysozyme and α-Lactalbumin. The data obtained with lysozyme and α-lactalbumin, which had been collected on an absolute scale over an angular range extending from $2\Theta = -5°$ to $+5°$, were treated in accordance with eq 1–11 above, as illustrated by the Guinier plot (eq 1; Figure 5) and the $s^3 j_n(s)$ *vs.* s^3 plot (eq 6; Figure 6) of lysozyme. Concentration plots of the resulting parameters are shown in Figures 7 and 8. No statistically significant difference is found for the values of molecular weight (lysozyme, 13,500 $\pm$ 300 *vs.* α-lactalbumin, 13,300 $\pm$ 600) and only a very slight, probably not significant, difference for the radius of gyration (14.3 $\pm$ 0.2 *vs.* 14.5 $\pm$ 0.2 Å), the molecular volume (24,200 $\pm$ 400 *vs.* 25,100 $\pm$ 800 Å), and the surface-to-volume ratio (0.25 $\pm$ 0.03 *vs.* 0.24 $\pm$ 0.02 Å^{-1}). Values for the electron density difference (0.078 $\pm$ 0.002 *vs.* 0.067 $\pm$ 0.002 electron/Å^3) and the degree of hydration (0.33 $\pm$ 0.02 *vs.* 0.37 $\pm$ 0.03 g H_2O/g protein) differ more significantly.

These findings are in essential agreement with the expectations raised by the homologous amino acid sequences of the two proteins and the consequent conjecture of conformational similarity. The sequence homologies are very substantial, extending to better than 30% of the chain and including the position of the four disulfide bridges (Brew *et al.*, 1967). Furthermore, the replacements of amino acid residues necessary to change the sequence of lysozyme to that of α-lactalbumin are conservative in that they can be accommodated by the same secondary and tertiary structure (Browne *et al.*, 1969). Small observed dissimilarities found in optical rotatory dispersion and circular dichroism (Aune, 1968; Kronman, 1968) and in nuclear magnetic resonance investigations (Cowburn *et al.*, 1970) were considered accounted for on the basis of dissimilar side chains. An immunological study (Strosberg *et al.*, 1970) detected a weak cross-reaction; immunological findings, however, resulting presumably from similar residues in exposed parts of the two proteins, cannot be conclusive regarding molecular conformation because of their limited significance with respect to the portions of the chain responsible for maintaining the three-dimensional structure. X-ray scattering, which gives

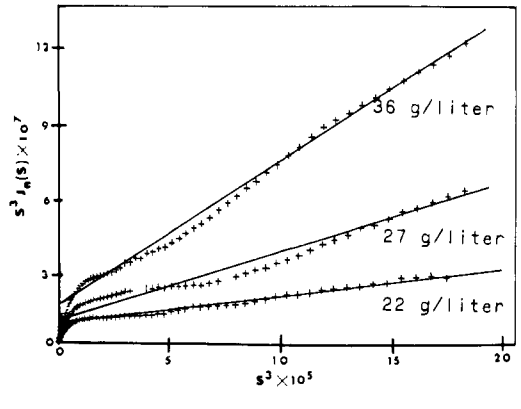

Figure 6. Computer plots of $s^3 j_n(s)$ *vs.* s^3 for lysozyme

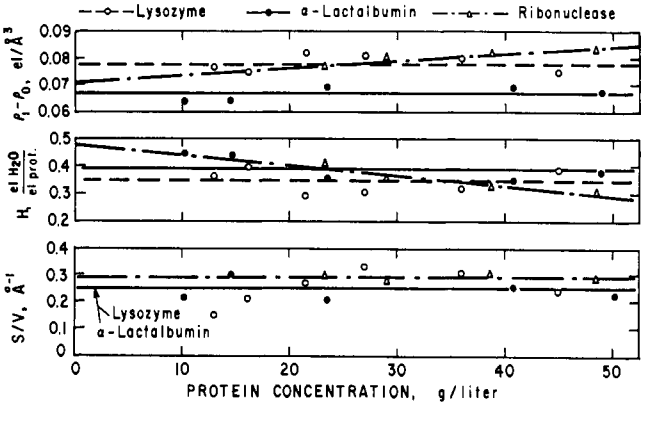

Figure 8. Concentration plots of $\Delta\rho$, S/V, and H for three proteins

more direct knowledge of conformation, furnishing at the same time independent information on molecular size and shape, would be more pertinent. An earlier small-angle X-ray scattering investigation (Krigbaum and Kügler, 1970) appears to have found some substantial differences between lysozyme and α-lactalbumin. In our work we have not been able to reproduce these differences. Thus we conclude that, except for a small difference in the extent of hydration, the two proteins have essentially identical macromolecular parameters. This result confirms the report of Browne *et al.* (1969) that the amino acid sequences of both proteins can be accommodated by the same three-dimensional folding of the polypeptide chains.

Ribonuclease. In view of the similarity of results obtained for lysozyme and α-lactalbumin, it could be suggested that X-ray scattering as a method might not be sensitive enough to distinguish between two globular proteins of very similar size. In this connection the results for ribonuclease, another globular protein of also approximately the same size, are of interest. Parameters calculated from data obtained in the same way for this protein, and likewise plotted in Figures 7 and 8, show a somewhat higher radius of gyration (14.8 ± 0.4 Å), a higher molecular weight (14,900 ± 800), a lower molecular volume (22,000 ± 700 Å^3), and a considerably higher degree of hydration (0.46 ± 0.04 g H_2O/g protein) and higher surface-to-volume ratio (0.29 ± 0.02 Å^{-1}) than either lysozyme or α-lactalbumin. The electron density difference for ribonuclease (0.071 ± 0.002 electron/Å^3) is intermediate between the values for the other two proteins. Also of interest is the distinct concentration dependence of nearly all these parameters (with the exception of the molecular volume and the surface-to-volume ratio) in the case of ribonuclease, whereas no significant dependence was observed for lysozyme or α-lactalbumin.

It is thus apparent that while no very substantial size or shape differences were observed between lysozyme and α-lactalbumin, significant differences may be seen between either of these proteins on the one hand and ribonuclease on the other. More extensive differences permitting more detailed conclusions would probably be detected on extending the studies to higher scattering angles (and, necessarily, higher concentrations), where intraparticle interference effects would be more notable and the shape factor would become of greater consequence.

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