REVIEW ARTICLE

MOLECULAR SIZE AND SHAPE

A Review of the Light-scattering Method applied to some Important Biological and other Macromolecules. Part I

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Introduction

Many important biological macromolecules have been studied in more detail in recent years as a result of the development of the light-scattering method. Researches using this technique have contributed not only to the elucidation of the size and shape of biopolymers, high polymers, hydrosols, aerosols, surface active substances and other high molecular weight compounds but also to the study of reaction mechanisms in a range of conditions wider than those obtainable by some other methods. Although substances having molecular weights of less than 1,000 have been examined, the light-scattering method is generally used for the study of macromolecules having weights in excess of 5,000.

Two important differences between solutions of large molecules and "true" solutions are the deviation from ideality and the methods of studying their kinetic and thermodynamic properties. Reliable and accurate information about the physical constants of macromolecules in solution is needed if the mechanism of their reactions is to be understood.

The physical characteristics of macromolecules in solutions as used for measurement by the light-scattering method, are often akin to those existing in natural conditions (*in vivo*) in biological systems, but the concentrations at which such measurements are made are necessarily small so that the results can be extrapolated to infinite dilution and interpreted in terms of thermodynamic functions. Comparison of information obtained by light-scattering under these circumstances with that obtained by other methods, where much higher concentrations and even materials in the dry state are often used, is not always justified.

Several methods for the determination of the molecular characteristics of large molecules are in general use; the classical methods, e.g., cryoscopy, ebulliometry and vapour pressure are often unsatisfactory because of the very high concentrations required to give a measurable effect. The more important methods and the properties on which they depend may be briefly examined.

1. *Osmotic pressure.* This is the most widely used method for the molecular weight range between 5,000 and 100,000. It gives a number average molecular weight and a thermodynamic measure of interaction between molecules (constant B) but no information about shape. Possible errors are attributable to irreversible adsorption of molecules on

membranes and high net surface charges, which set up a membrane potential.

2. *Viscosity.* The method gives a molecular weight approximating to weight average. A model is required (i.e., equations for rods, coils or spheres) to interpret the shape of the molecule which may be difficult to define owing to distortion by the applied force.

3. *Sedimentation, velocity and diffusion constant.* These methods are complementary and depend on the frictional properties and sizes of the molecules. They give a weight average molecular weight and information about size and shape. They are valuable for resolution of a polydispersed, non-interacting system into several components.

4. *Dipole moment and relaxation constant.* This method gives information on dipole-ion structure (e.g., of amino acids and peptides) and distribution of surface charges. It is limited to work in solutions of low electrical conductivity.

5. *Ultrafiltration.* Using membranes of different grades the method gives an approximation of size.

6. *Surface films.* Under favourable conditions, this method gives molecular weight, mode of orientation and molecular surface area at an interface. It can be extended to obtain the effects of pH, oxidants, and the presence of electrolytes, on physical and chemical characteristics.

7. *Chemical.* This is by determination of equivalent weight by using gravimetric analysis or by treatment with a compound capable of reacting with certain free groups in the molecule. Thus, Sanger's dinitrofluorobenzene reagent reacts with amino groups of a protein.

8. *Electron microscope.* The specimens are studied in high vacuum and therefore in a dry state. The molecules are generally required to have dimensions greater than 100 Å. It is difficult to distinguish small structures from background material. This method gives visual evidence of size and shape.

9. *Direct particle counting.* This is effected by counting a weighed number of particles in a known volume of solution; a reference substance is required. Molecular weight only is given.

10. *X-ray diffraction.* The method is to obtain crystallographic unit cell measurements of highly ordered systems. It gives information on molecular size, shape and solvation. The structures of complex proteins and nucleic acids have been examined extensively in this way.

11. *Light-scattering.* A revival of interest in light-scattering as a means of investigating colloidal particles in solution began with the papers of Debye[1,2]. Suitable theoretical treatments were developed and the practical difficulties were studied in many laboratories. The method has been valuable in determining the physical constants and the kinetic and thermodynamic properties of large molecules in solution. It has contributed information on particles of much greater molecular weight ($M_w > 1{,}000{,}000$) and a wider range of temperatures and ionic strengths

of solutions than is possible by osmometry. It can also be used to determine the rates of changes in size and shape when aggregation or disaggregation in solution takes place since it gives instantaneous results; kinetic effects can thus be studied as a function of time. Systems that are deformed by shear or affected by disturbance can be studied to advantage by this method.

Although the theory and practice of light-scattering have been extending rapidly, the quantitative interpretation of results is mainly concerned with the macromolecules in a monodisperse state in dilute solutions, whereas many systems are polydisperse. The light-scattering method measures the weight average molecular weight (M_w) and in a polydisperse system the results are always higher than, for instance, those obtained from osmotic pressure measurements which give a number average molecular weight (M_n). For asymmetric particles the dissymmetry method, where measurements are taken under equilibrium conditions, is better than the streaming birefringence and viscosity methods (dynamic) which depend on setting up shear gradients with a consequent distortion of the particle shape. Cleverden, Harvey, Laker and Smith[3] describe a statistical treatment (random flight) to show a close similarity between viscosity and light-scattering.

Simplified Theory of Light-scattering

When a beam of light passes through a material the electric field associated with the beam induces periodic oscillations in the molecules of the material; the molecules thus become oscillating dipoles which serve as a source of secondary radiation (scattered light) with a wavelength equal to the incident beam. The small fraction of secondary radiation emitted at a different wavelength (Raman effect, Compton effect and fluorescence) is neglected in the theory. The intensity of this scattered radiation increases with the size of the molecules and the readiness with which they respond to the induced periodic oscillations (called the polarisability). If the molecules have a maximum dimension of less than 1/20th the wavelength of the incident beam they are regarded as single dipoles and are called Rayleigh scatterers. Larger molecules are regarded as a fixed array of point sources in which case the light scattered from one part of the molecule may be out of phase with that coming from another, resulting in destructive internal interference and a lower scattering intensity.

The problem of scattering divides itself into two main categories.

1. Scattering of light from solutions of small molecules.

2. Scattering of light from solutions containing molecules comparable in size with the wavelength of light.

1*a*. *Small Isotropic Particles*

Light scattered by small particles randomly disposed is measured experimentally as the "reduced intensity" (sometimes called the Rayleigh ratio) denoted by R_θ. If I_0 is the intensity of the incident beam of

unpolarised light, i_θ the scattered intensity per unit volume at an angle θ to the incident beam, and r the distance between the observer and the scattering system; then:

$$R_\theta = \frac{i_\theta r^2}{I_0} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

For an unpolarised incident beam the angular dependence of R is given by the relation:

$$R_\theta = R_{90}\,(1 + \cos^2\theta) \quad .. \quad .. \quad .. \quad (2)$$

The scatter is therefore symmetrical about 90° to the incident beam. At 90° the scattered light is completely vertically plane-polarised, the polarisation decreasing on either side of 90°, finally becoming zero at 0° and 180°. Figure 1 shows the relative intensity of scattering [$(1 + \cos^2\theta)$ term] about a small isotropic particle.

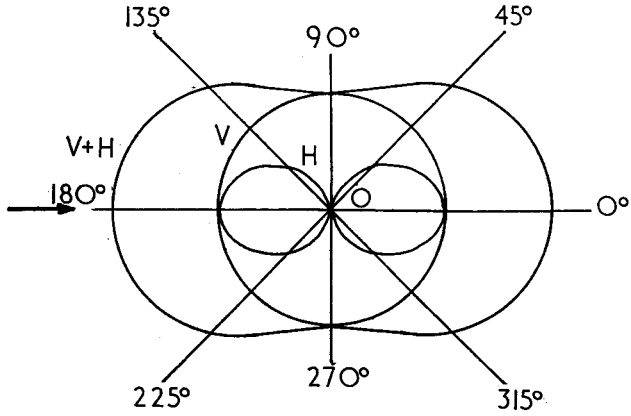

FIG. 1. Angular distribution of scattering of light $(1 + \cos^2\theta)$ by a small isotropic particle at O. V corresponds to the vertically polarised component (the unity term) and H to the horizontally polarised component (which varies at $\cos^2\theta$).

The intensity of light scattered by ν isotropic particles per unit volume of a gas is given by the Rayleigh expression:

$$R_\theta = \frac{8\pi^4 \nu\alpha^2}{\lambda_o^4}(1 + \cos^2\theta) \quad .. \quad .. \quad .. \quad (3)$$

where α is the polarisability and λ_o is the wavelength of light in vacuum.

1b. Scattering from Dilute Solutions

If the molecules are in a highly ordered state as, for instance, in a perfect crystal, total destructive interference takes place and no light is scattered. Pure liquids are ordered, at least compared with the randomness of solute molecules in solution. Smoluchowski[4] explained the scattering in pure liquids by the theory of thermodynamic fluctuations. Molecules of a liquid are in continuous Brownian movement and it is conceivable that within a small element of volume the number of molecules within it, i.e., the density of this element, will vary continuously. In terms

of the whole liquid, density fluctuations occur and the greater the Brownian movement the greater the fluctuations and also the scattering. In solutions, inhomogeneities will be caused by density fluctuations and also by fluctuations in concentration of solute molecules.

Assuming that the solute molecules are large compared with the solvent molecules, equation (3) can be used to obtained a relation between R_{90} (corrected for solvent effects) and the molecular weight of a dissolved macromolecule. The polarisability term (α) is related to dielectric constant (ϵ) which in turn can be put in terms of a measurable quantity called the specific refractive index increment $\left(\frac{n - n_o}{c}\right)$ where n and n_o are the specific refractive indices of the solution of concentration c and pure solvent, according to the relations:

$$4\pi\nu\alpha = \frac{\epsilon - \epsilon_o}{\epsilon_o} = \frac{n^2 - n_o^2}{n_o^2}.$$

Replacing ν by $\frac{N_o c}{M}$ where N_o is Avogadro's number, equation (3) becomes:

$$R_{90} = \frac{2\pi^2 n_o^2 (n - n_o/c)^2}{N_o \lambda_o^4} \; cM \quad .. \quad .. \quad (4)$$

or

$$R_{90} = KcM \quad .. \quad .. \quad .. \quad (5)$$

K is a constant for a given solution. By measuring the scattered light at dilute concentrations the molecular weight of the solute can be found.

Alternatively, the scattering may be expressed in terms of diminution in intensity of the incident beam as it passes through the solution. If the intensity is diminished from I_0 to I on traversing a path length l in the solution, then $I = I_0 e^{-\tau l}$. Integrating for scattering ($i\theta$) over the surface of a sphere gives the result:

$$\tau = \frac{8\pi}{3} R_0 = \frac{16\pi}{3} R_{90} \quad .. \quad .. \quad .. \quad (6)$$

Equation (5) can now be put in the form:

$$\tau = HcM \quad .. \quad .. \quad .. \quad .. \quad (7)$$

where H is the constant for a given solution. Both equations can be used in light-scattering measurements but usually the reduced intensity is preferred since the turbidity of many solutions is very low and difficult to measure accurately.

1*c*. *Higher Concentrations*

The plot of turbidity: concentration for aqueous sucrose solutions[5] (Fig. 2) shows that at higher concentrations the relationship of equation (7) breaks down. Since the particles are closer together they cease to be independent scatterers and destructive external interference results. In solutions at higher concentrations there will be local fluctuations of the concentration of the solute as well as local fluctuations of the solvent.

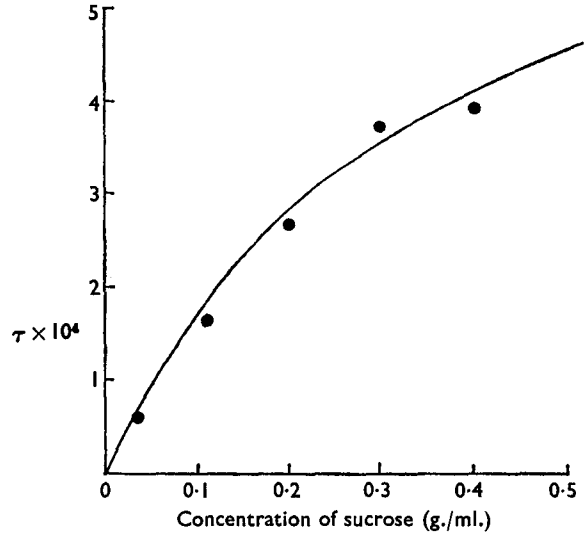

FIG. 2. Variation of turbidity with concentration of an aqueous solution of sucrose (after Debye, *J. phys. Chem.*, 1947, **51**, 18, with permission).

To this additional fluctuation Einstein applied the theory of thermodynamic fluctuations.

Einstein directly related the scattering of light to the osmotic pressure (P) of non-ideal solutions in the Smoluchowski-Einstein equation:

$$\tau = \frac{HRc}{\partial P/\partial c} \quad .. \quad .. \quad .. \quad .. \quad (8)$$

Debye transformed equation (8) into a workable form by resolving the term $\partial P/\partial c$ using the osmotic pressure relation:

$$\frac{P}{RT} = \frac{c}{M} + Bc^2 \quad .. \quad .. \quad .. \quad .. \quad (9)$$

where B is called the interaction constant.

Equation (8) then becomes:

$$\frac{Hc}{\tau} = \frac{1}{M} + 2Bc \quad .. \quad .. \quad .. \quad (10)$$

and also

$$\frac{Kc}{R_{90}} = \frac{1}{M} + 2Bc \quad .. \quad .. \quad .. \quad (11)$$

By taking a series of readings of R_{90} at different concentrations a linear plot is obtained having an intercept equal to the reciprocal of the weight average molecular weight and a slope equal to $2B$.

1*d*. *Anisotropic Particles*

Quite often the particles, although small, are anisotropic. In this case the induced electric moments tend to be of different magnitude in

different directions depending on the shape of the particle. Consequently the 90° scattering will not be completely vertically polarised; as a result the scattering intensity at 90° will be greater than that for an isotropic particle and the Cabannes factor[6] must be applied.

2. *Large Particles*

If any dimension of the macromolecules exceeds $\frac{\lambda}{20}$, the Rayleigh concept of scattering from a single dipole breaks down. Larger particles can be regarded as a fixed array of single dipoles, radiating wavelets in different phases, destructively interfering with each other; multipolar electric and magnetic fields are also set up within the particle. Moreover, the magnitude of interference effects increases with the scattering angle.

Essentially the limiting form of equation (11) for all angles of scattering can be retained by introducing a particle scattering factor $P(\theta)$ defined by the equation:

$$P(\theta) = \sum_i \sum_j \frac{\sin ksr_{ij}}{ksr_{ij}} \quad .. \quad .. \quad (12)$$

where r_{ij} is the distance between two scattering element i, j, $k = \frac{2\pi}{\lambda}$ (where λ = wavelength of light in solution) and $s = 2\sin\frac{\theta}{2}$.

The values of $P(\theta)$ for a sphere (Rayleigh[7], Gans[8]) of diameter D, a rod (Neugebauer[9]) of length L, and a coil (Debye[5] and also Zimm, Stein and Doty[10]) having a root mean square value of distance between its ends R, are given by the expressions:

Uniform spheres: $$P(\theta) = \frac{3}{x}\left[(\sin x - x\cos x)\right]^2 \ldots x = \frac{ksD}{2} \quad .. \quad (13)$$

Rods: $$P(\theta) = \frac{1}{x}\int_0^{2x} \frac{\sin w}{w}\, dw - \left(\frac{\sin x}{x}\right)^2 \ldots x = \frac{ksL}{2}$$

Coils: $$P(\theta) = \frac{2}{x^2}\left[e^{-x} - (1 - x)\right] \ldots x = \frac{(ksR)^2}{6}$$

In practice, $P(\theta)$ can be obtained in either of two ways: (*a*) by the dissymmetry method[5,10], (*b*) by the simultaneous angle and concentration extrapolation of Zimm[11].

The dissymmetry method requires a model. The dissymmetry, previously calculated as a function of some absolute dimension of the model, is measured experimentally and the dimension of the particle determined. For a given angle the dimension gives a value for x in equation (13) and enables $P(\theta)$ to be calculated.

The extrapolation method of Zimm does not require a model. Measurements of R_θ for a wide range of angles and different concentrations are

plotted $\left(\text{as}\frac{Kc}{R\theta}\right)$ as a function of $\sin^2\frac{\theta}{2} + kc$ (k an arbitrary constant). Extrapolation along lines of constant concentration to zero angle and constant angle to zero concentration gives an intercept equal to the reciprocal of the molecular weight and a particle scattering factor of unity (after correcting for depolarisation).

So far particles with a relative refractive index (relative to the medium) approaching unity have been discussed. When the relative refractive index is large the scattering pattern is complicated and it is only for spheres that formulae have been developed. Anticipating that the former systems are studied by workers in pharmaceutical chemistry, the reader is referred to Mie's[12] original paper for his calculations on spheres having a high relative refractive index. In multicomponent systems scattering of light will be further complicated by the different refractive index of each component. Treatment of this problem is mentioned in Part II (references 195, 196 and 197).

Practice

The experimental work can be divided into two main parts.

1. Measurement of the reduced intensity and depolarisation of scattered light.

2. Measurement of the refractive index difference between solution and pure solvent.

Reduced Intensity of Scattering

There are variations in design of apparatus for measuring the light scattered by systems of low turbidity but the essential requirements are a source of monochromatic light collimated to give a powerful convergent or parallel incident beam, a glass cell containing the solution, a receiver (usually a 9- or 11-stage photomultiplier) and its signal strength detector (a galvanometer).

Measurements at 90° to the incident beam are inadequate for most work and instruments are adapted to measure scattering over a range of angles on either side of 90°. In the simplest arrangement, the receiving photomultiplier is itself moved, this reduces the optical path lengths of incident and scattered beams to a minimum. The disadvantages are in moving a bulky unit connected to high tension power lines and the large arc described over the range of angles. Alternative designs have a fixed phototube and moveable optical systems which may vary the angle of the incident beam entering the light-scattering cell (see Peaker[13]) or carry the scattered light through a "rotating periscope" into the detector (see M'Ewen and Pratt[14]).

The author uses the apparatus shown in Figure 3, which is described elsewhere[15] and is based on the "Universal" light-scattering apparatus designed at the Colloid Science Department, University of Cambridge[16]. This instrument, elegant in its optical simplicity, enables measurements to be taken every few degrees over a wide range of angles of scattering.

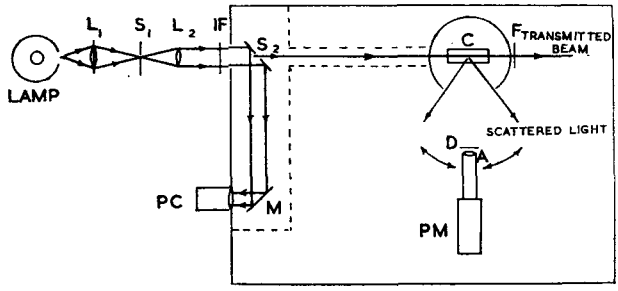

FIG. 3. Diagrammatic sketch of apparatus.

L_1 L_2 = Lenses. S_1 S_2 = Slits.
IF = Interference filter isolating $\lambda = 4358$Å.
F = Neutral density filter.
D = Polaroid disc for measuring depolarisation of scattered light.
A = Aperture. M = Mirror .
C = Cell immersed in thermostat-jacket.
PC = Photocell (connected via switch to galvanometer) for measuring intensity of incident beam.
PM = 11-stage photomultiplier (connected via switch to galvanometer) for measuring intensity of scattered light.

This apparatus has also been adapted by Ottewill and Parreira[17] to follow reaction kinetics. It seems to the writer that the electronics in the "Universal" instrument could be much simplified by use of an 11-stage photomultiplier.

In all light-scattering measurements the elimination of stray scatter is essential to obtain a perfectly linear angular light-scattering envelope. The system must be freed from stray radiation by blackening the surfaces surrounding the cell which contains the solution and also the receiving system, both of which are contained in a light-tight box.

Clarification

The problem of clarification of solutions, particularly when aqueous, is a formidable one. The exclusion of dust, easily seen as bright points when solutions are viewed in the position for small angle scattering, is essential. An all-enclosed glass distillation apparatus, sintered glass or sintered platinum[18] filters, Elford membranes[19], and chemical[20] (precipitation) methods of purification have been described. For aqueous systems, ultrafiltration is probably the most effective but the writer has found that centrifugation and the use of Nos. 4 and 5 sintered glass filters have produced results comparable with other workers.

Calibration

Since the incident and scattering intensities of light are of widely different orders of magnitude, measurement of these to obtain the reduced intensity or Rayleigh's ratio $\left(R_{90} = \frac{i_{90}r^2}{I_0}\right)$ is difficult and most instruments are calibrated.

Several calibrating liquids are in general use. Solutions of polymers (e.g., polystyrene[21] and polymethylacrylic acid[22]) have been found suitable but these substances require careful fractionation and standardisation by other methods. The writer has used a Ludox solution (a colloidal dispersion of spherical silica particles) and benzene and toluene, the organic solvents being preferred for the reasons suggested by Cleverdon, Harvey, Laker and Smith[23]. Corning optical glass and Perspex are used by the writer as secondary standards (suggested by the workers at Cambridge) to check the apparatus at frequent intervals. Correction factors for optical effects of instrumentation are necessary (see Stacey[24]).

Depolarisation

When the incident beam is to be polarised or when measuring depolarisation of the scattered light, polaroid discs with an edge parallel to one axis of transmission are generally used. The presence of dust, secondary scattering and other optical causes can result in false high values of depolarisation. It has been found[25] that photocathodes are not always equally sensitive to light falling upon them in different planes.

Refractive Index

In the Debye interpretation of light-scattering (equation 4) the refractive index difference between solution and pure solvent appears as a squared term and therefore its precise experimental determination is important. Its value is generally of the order 10^{-3} and to obtain an accuracy of 1 per cent difference readings of considerably higher order (approaching 10^{-6}) are necessary. The most suitable instruments are interference refractometers. An adaptation of the Rayleigh interference refractometer for use with monochromatic light is suggested by Grunwald and Berkowitz[26].

Debye[27] and Brice, Halwer and Speiser[28] have designed simple differential refractometers for this purpose.

Accuracy of the Method

The accuracy of light-scattering measurements is largely dependent on the substance investigated and the extent of dilution to which reliable readings can be made, providing the necessary precautions are taken. For dilute solutions of small particles the accuracy would be greater than that at higher concentrations and with larger asymmetric molecules. For most substances which show a tendency to aggregate, for example, proteins and surface-active substances, an accuracy of ± 10 per cent should be possible. Substances that show high dissymmetry would be of a similar order since measurements at low angles of scattered light relative to the incident beam are susceptible to inaccuracies because of the presence of dust.

The International Union of Pure and Applied Chemistry recently organised an investigation on the characterisation of fractions of polystyrene by the light-scattering and other methods. A summarised report by Mark and Frank[21] on a typical sample of polystyrene in toluene showed that errors for the four methods, viz., viscosity, osmotic pressure, light-scattering and sedimentation-diffusion were 1·5, 23·9, 7·5 to 9·0 and

6·6, respectively. The viscosity and ultracentrifuge results were reported as satisfactory, the light scattering discrepancies were attributed to unsatisfactory calibration of the instruments (though consistency on the light-scattering values between the various investigators for the molecular weight and radius of gyration was satisfactory in the writer's view) and the errors in osmometry arising from the unsatisfactory performance of the semi-permeable membranes.

The best values from light-scattering measurements are probably within ± 5 per cent.

Applications

Biopolymers and Substances Having Physiological Activity

Light-scattering has made a valuable contribution to the physical characterisation of many biological macromolecules and to studies of the reaction mechanisms which involve changes in molecular weight and other properties.

Some of the more interesting studies are reviewed.

Insulin

On account of its physiological importance insulin has been characterised in detail by many methods. In earlier work discrepancies in the estimates of its molecular weight existed but more recent investigations have led to accurate values being determined.

Molecular weights of 35,000 are now known to be those of aggregates. In 1938 Crowfoot[29] gave a value of 36,000 by X-ray diffraction which clearly indicated from the symmetry of the pattern that the crystals were composed of three sub-units, each of molecular weight 12,000. Detailed amino acid analysis by Sanger[30] in 1949 indicated a minimum molecular weight of either 6,000 or 12,000. More recent investigations by light-scattering[31] give evidence that the minimum molecular weight in aqueous solution is 12,000, which corresponds to a strongly hydrogen-bonded dimer; the reversible association of the protein, the configuration of the aggregates and related thermodynamic properties have since been studied.

Steiner[32] investigated the effect of pH on the tendency of the zinc-insulin to dissociate with increasing dilution (Fig. 4). The curves showed that, at constant ionic strength, dissociation was favoured by an increasing positive charge but the limiting value was the same, giving a minimum molecular weight of 13,000. Increasing the ionic strength was shown to favour association as expected. From these results Steiner considered the dissociation to be stepwise and calculated two dissociation constants for pHs 1·52 and 2·12, indicating trimer formation. At the two higher pH values indications of some association to pentamer formation were apparent. Further measurements at different temperatures gave values for the change in heat content and entropy change associated with the equilibrium states. Similar observations in substantial agreement with Steiner have also been reported by Doty and Myers[33].

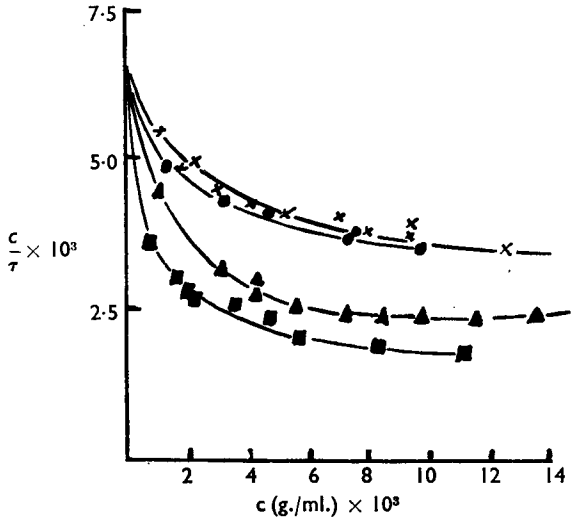

FIG. 4. Dissociation of Zn-insulin on dilution in 0·2M KCl at four different pH values (after Steiner, *Arch. Biochem. Biophys.*, 1952, **39**, 333, with permission).
● pH = 1·52 × pH = 2·12 ▲ pH = 2·65. ■ pH 3·13.

Serum Albumins

Several serum albumins, including horse, pig, human, and bovine have been investigated in some detail by the light-scattering and other methods. Their behaviour in acid solutions, interaction effects in globulin solutions and the influence of salts on the size and shape of many complexes containing a serum albumin component have been examined. The effects of denaturation of serum albumins on their tendency to swelling and aggregation have also been reported.

Results obtained with bovine serum albumin have been found to be reproducible and reliable; this and similar proteins have therefore frequently been used in experiments to study theoretical developments. Doty and Steiner[34, 42] predicted the behaviour and interactions of charged macromolecules and subsequently confirmed their theory experimentally using bovine serum albumin (BSA). The Kirkwood-Shumaker[35] theory was also confirmed experimentally using bovine plasma albumin by Timasheff, Dintzis, Kirkwood and Coleman[36]. It appears that the molecular weights of the different serum albumins obtained by the light-scattering method are generally slightly higher than those obtained by other methods. BSA has probably received most attention and for this substance sedimentation and diffusion studies by Creeth[37] gave molecular weights of 65,400 and 69,000, while osmotic pressure measurements by Scatchard, Batchelder and Brown[38] gave the value of 69,000. Using an absolute photoelectric turbidimeter, a minimum absolute molecular weight of 73,000 for BSA and 79,000 for horse serum albumin were reported by Halwer, Nutting and Brice[39]. They found that the

molecular weight and moisture content of all samples increased by varying amounts with time.

A comparative study of BSA in pure water and 0·15M sodium chloride was made by Danliker[40] to examine the accuracy of measurements on proteins in the absence of supporting electrolytes. By removing salts on mixed-bed ion exchange columns and clearing aggregates by high speed centrifuge, he obtained a molecular weight value for BSA in pure water in good agreement with other methods. The molecular weight in the salt solution was the same as that in pure water within experimental error. In the presence of sodium chloride it was suggested that aggregates were dissociated and were not removed by centrifugation. Although agreement for the molecular weight of the protein in the two solutions was close, the slopes of the reciprocal scattering: concentration curves (Fig. 5) showed a small positive slope for BSA in sodium chloride and a large negative slope for BSA in pure water. The theory of Kirkwood and Shumaker predicted that intermolecular, attractive forces operated over a long range resulting in a negative excess chemical potential for the isoionic BSA in pure water.

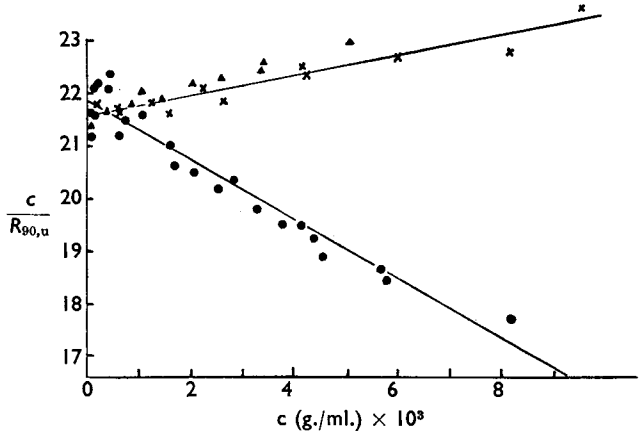

FIG. 5. Light-scattering of bovine serum albumin (after Danliker, *J. Amer. chem. Soc.*, 1954, **76**, 6036, with permission).
○ Centrifuged and measured in water.
× Centrifuged and measured in 0·15M sodium chloride.
▲ Centrifuged in water and measured in 0·15M sodium chloride.

Edsall, Edelhoch, Lontie and Morrison[41] discussed the effects of net proton charge per molecule of BSA and the ionic strength of the solution on the interaction constant B. Measurements at several different concentrations of various salts compared favourably with similar values from osmotic pressure measurements. Better reproducibility, however, could be expected from the light-scattering method (compared with osmotic pressure) where difficulties such as establishing equilibrium between phases across a membrane in the presence of molecules carrying a high net positive charge and at very low ionic strengths, do not arise.

Doty and Steiner[34, 42] observed that, in salt-free acid solutions, the scattering intensity and angular distribution of BSA particles differed considerably from previous observations on this protein. In acid the charged protein molecule becomes surrounded by an electric double layer giving the molecule an effective diameter considerably greater than its molecular diameter, hence interaction between the molecules is then characterised by a distance of closest approach. Due to electrostatic charges on the particles, long-range intermolecular repulsive forces come into play and the particles can no longer be regarded as independent volume elements. External interference of light consequently takes place, resulting in a diminished intensity of scattered light. From their results illustrated in Figure 6 it can be seen that the change in reciprocal scattering

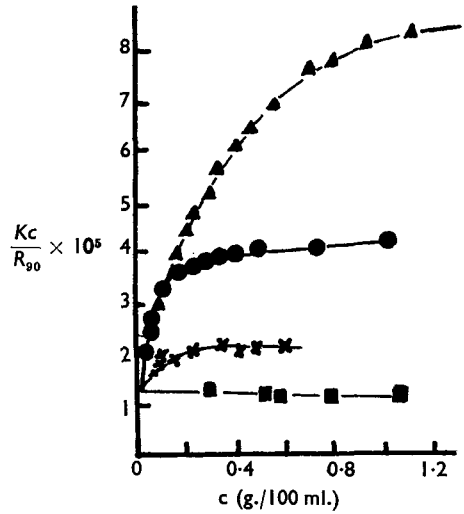

FIG. 6. Variation of $\frac{Kc}{R_{90}}$ with concentration of bovine serum albumin. Deviations from ideal behaviour increase with increasing charge on the particle (after Doty and Steiner, *J. chem. Phys.*, 1949, **17**, 743; 1952, **20**, 85, with permission).

▲ pH = 3·30. ○ pH = 4·10. × pH = 4·36. ■ = pH 5·10.

intensity (Kc/R_{90}) with increasing concentration clearly depends on the charge, the behaviour at pH 3·3 showing a marked deviation from ideality, whilst at pH 5·1 when the protein is isoelectric the change in reciprocal scattering intensity almost vanishes. The increased deviation from linearity with change in pH within the acid range itself is attributed to the change in size of the double layer which varies as the inverse cube of the concentration of gegen ions.

From depolarisation of fluorescence measurements, Weber[43] suggested that the BSA molecule dissociated at pH 1·86 into two sub-units of approximately equal molecular weights. Later Harrington, Johnson and Ottewill[44] carried out further light-scattering studies and found that BSA had a constant molecular weight of 72,000 from pH 4·5 to pH 1·9

(the dissymmetry showing a small change between 1·05 and 1·09); their results from sedimentation velocity also showed no change in molecular weight.

The extent of the process of denaturation of a protein on its physical characteristics (solubility, size and shape, hydration, and electrophoretic phenomena all change to some degree) have been investigated but conclusions are indefinite. The increase in viscosity and decrease in the value of the diffusion constant found when BSA is dissolved in concentrated urea solutions has been attributed to an unfolding or uncoiling of the main polypeptide chains from their native configuration. Doty and Katz[45], however, could not find any increase in dissymmetry of scattered light indicative of such a change. Variation of the pH of the solutions showed that preferential adsorption of water took place on the acid side of the isoelectric point (3,000 molecules of water preferentially bound at pH 3) and preferential adsorption of urea on the alkaline side of the isoelectric point (2,000 molecules urea preferentially bound at pH 8) —neither water nor urea were preferentially bound at the isoelectric point itself. Evidence from these studies led Doty and Katz to the conclusion that the essential change taking place within the BSA molecule is one of isotropic swelling.

In 1950 Greenstein and Hoyer[46] reported that, under special conditions, sodium thymus nucleate prevented the heat coagulation of serum albumin —nearly complete protection of the albumin solutions against heat coagulation was obtained by as little as one part of sodium thymus nucleate to 200 parts of BSA in salt-free solutions.

Geiduschek and Doty[47] investigated the complex formation in solution between BSA and deoxyribonucleic acid (DNA) by light-scattering at room temperature. They mixed the BSA and DNA together and measured the light-scattering by diluting this mixture into a phosphate buffer as solvent. At pH 7·47 and 6·46 no reaction was observed but at pH 5·51 and ionic strength 0·1 DNA was found to bind 35 per cent of its own weight with BSA in a 1 : 1 mixture, i.e., on an average each molecule of nucleic acid bound 11 ± 2 molecules of BSA with no appreciable change in shape of the nucleic acid. Geiduschek and Doty reasoned that, for the protection of BSA by such a small amount of sodium deoxyribonucleate used by Greenstein and Hoyer, the albumin must have been in continuous molecular contact with the nucleic acid. This corresponded to about one serum albumin molecule per nucleotide, representing extremely tight binding in contradiction to the relatively weak interaction shown experimentally by Geiduschek and Doty. From observations of Ambrose and Butler[48] they concluded that strong binding was possible when parts of the protein structure were made accessible by denaturation. The search for a stronger type of binding was subsequently made by Zubay and Doty[49].

In preliminary experiments on individual components, Zubay and Doty showed by light-scattering that the extent to which BSA coagulates was largely dependent on pH or ionic strength. Coagulation was favoured by keeping the negative charge on the albumin at a minimum by either

adjusting the pH to the isoelectric point (4·5) or by the presence of electrolytes. At higher pH values the negative charge rose, electrostatic repulsion between molecules increased and effective contact between them was prevented. No degradation of the DNA took place under the required conditions. When the pH of a mixture having a weight ratio BSA : DNA of 15 was controlled (as in the coagulation of BSA alone) the reaction went to completion, that is, complete binding took place below pH 5, thereby preventing self-aggregation of the serum albumin. Zubay and Doty calculated that at least 1,800 molecules of serum albumin could be bound per DNA molecule. Since the absence of gelation and the presence of a single peak in the ultracentrifuge indicated that there was no cross-linking, the physical characteristics were investigated. The molecular weight determinations by light-scattering were in close agreement with the value calculated, confirming the interpretation of the reaction. Addition of 8M urea to the complex reduced the light-scattering intensity drastically, suggesting that the binding was mainly due to hydrogen bonding.

Lysozyme

Lysozyme was shown to be an enzyme by Meyer, Palmer, Thompson and Khorazo[50] and by Epstein and Chain[51], who suggested that when lysozyme acted on a specific carbohydrate component of cell walls of susceptible micro-organisms it disrupted the cells. The ease with which lysozyme can be obtained in a pure crystalline tetragonal and orthorhombic form lent its readiness to X-ray examination from which a molecular weight of 13,900 was calculated[52]. Values for the wet lysozyme in the orthorhombic and tetragonal crystals was approximately 22,000, indicating about 54 per cent hydration.

The first value for the molecular weight of lysozyme (18,000) was obtained by Abraham[53] in 1939 by ultracentrifugation. Osmotic pressure measurements[54] gave values of 14,700 and later 17,500. Amino acid analysis has also been used, giving molecular weights of 14,700[55] and 14,900[56].

The light-scattering method is capable of giving satisfactory values for the molecular weight of proteins providing that a sufficiently pure and unchanged sample is available. Since lysozyme meets these requirements, its study by the light-scattering method is preferable to osmotic pressure, where, for such small molecules, it is difficult to obtain a semi-permeable membrane which will retain the protein without being so sluggish as to be impractical. Halwer, Nutting and Brice[39] studied this protein in 0·1M sodium chloride at pH 6·2; correcting for a depolarisation factor of 0·03 they gave a molecular weight of 14,800, which was unaltered after centrifuging for one hour at 125,000 *g*. A higher value (15,500) in 0·1M sodium chloride was obtained by Hughes, Johnson and Ottewill[57].

Steiner[58] made a quantitative study by light-scattering of the reversible electrostatic complexing of the two oppositely charged proteins lysozyme (egg white lysozyme has an isoelectric point of approximately 10·9) and bovine serum albumin (isoelectric point of approximately 5·3), and

found that interaction took place at pH values intermediate to the isoelectric points of the proteins; his results also showed that interaction was less as the ionic strength of the solution was increased. Steiner[59] has extended the quantitative aspect of the reversible association processes of globular proteins where association of a single molecule at any instant takes place. He developed equations from information obtainable by the light-scattering and osmotic pressure methods whereby the consecutive association constants of protein associations could be calculated.

Collagen

This protein, regarded as the most prevalent single organic constituent of animal organisms, is essentially a long, thin, partially crystalline, microscopically visible fibre.

In 1927 Nageotte[60] demonstrated that collagenous tissue elements were dispersible in acid solution but on neutralisation or addition of salts the material was reconstituted into an insoluble fibrous or gelatinous state. Since then both the soluble dispersions and the reconstituted fibrous precipitates have received much attention.

Low angle X-ray diffraction studies by Bear[61] and Hall, Jakus and Schmitt[62] showed a filament having a repetitive structure of fibrils. Bear suggested that the significance of this periodic structure may be regarded as either that the primary valency connection of the fibril unit is interrupted at regular intervals to provide a linear string of molecules, or the structure is a repeated pattern of amino acid residues extending along an indefinitely long polypeptide chain.

Bresler, Finogenov and Frenkel[63] studying an homogeneous extract of soluble rat skin collagen in citrate obtained a molecular weight of 70,000 $\pm$ 3,500 by sedimentation and diffusion and dimensions corresponding to a cylinder 380 $\times$ 16·7 Å. The authors suggest that this shape must appear coiled since the degree of polymerisation per residue of 4 Å was calculated to be about 600, which indicates a total polypeptide chain of approximately 2,400 Å.

Osmotic pressure measurements by Matthews, Kulonon and Dorfman[64] gave a number average molecular weight of 74,000 but the authors thought that large aggregates were probably present which made their estimates low compared with those obtained by other methods.

Several recent investigations by light-scattering have been made; most of them giving values for the molecular weight higher than 70,000. In 1954 Gallop[65] investigated an acid-soluble collagen (ichthyocol) from carp swim bladders by sedimentation, viscosity and light-scattering. He concluded that acid dispersions were long, thin, filamentous particles called "protofibrils" (observed previously by M'Ewen and Pratt[14]). His viscosity and sedimentation data gave a tentative axial ratio of the long, filamentous particle about 190 Å assuming it to be extended, but Gallop pointed out that a partial random coil shape was more probable. The mass per unit length was 88 avograms (one gram mass divided by Avogadro's number, see *Chem. Engng News*, 1950, **28**, 1841), that is, approximately one amino acid residue per ångström of particle contour length.

Since a residue can extend up to 3·67 Å along a contour axis, Gallop reasoned that the acid-dispersed particles contained up to three separate polypeptide chains in lateral aggregation. This is in agreement with Bear[66], who suggested that one residue occupies about an ångström of protofibril, the residues being connected end-to-end to form a single regularly coiled polypeptide chain capable of extension to many times its length. By light-scattering the acid-dispersed particles had an effective length of 4,100 Å and a contour length of about 13,400 Å, which is equal to about twenty times the disc-like repetitive structural unit in fibrils observed by electron microscope and small angle X-ray diffraction. From the mass:length value obtained by sedimentation and viscosity measurements Gallop calculated that the protofibrils would contain 50,000–80,000 avograms having a mass:unit length of approximately 120 avograms. He obtained a light-scattering molecular weight of $1{\cdot}67 \times 10^6$ for the protofibril which was somewhat less than the value of M'Ewen and Pratt (3 to 10×10^6) but enormously greater than the result obtained by Bresler and others. In addition, Gallop showed that on heating to only 40° at pH 3·7 the material underwent an irreversible change yielding a product called parent gelatin which had a light-scattering and sedimentation-diffusion molecular weight of nearly 70,000.

At first it may appear that on account of the mild treatment of collagen required to produce the parent gelatin it was the latter substance that gave Bresler his low molecular weight value of 70,000. Although such a large discrepancy existed in the light-scattering molecular weights, the intrinsic viscosities of these samples remained constant.

Boedtker and Doty[67] reinvestigated the problem of the molecular weight and dimensions of soluble collagen in 1956 by several physico-chemical methods; some of their results are tabulated below.

SUMMARY OF MOLECULAR CONSTANTS OF ICHTHYOCOL COLLAGEN
(after Boedtker and Doty, *J. Amer. Chem. Soc.*, 1956, **78**, 4267, with permission)

Method	Molecular weight	Length Å	Diameter Å
Osmotic pressure	310,000	—	—
Light-scattering	345,000	3,100	12·8
Intrinsic viscosity and molecular weight	—	2,970	13·6
Sedimentation and viscosity	250,000	—	12·0
Flow birefringence and viscosity	350,000	2,900	13·3

Attributing the discrepancies between constant values of the hydrodynamic characteristics and the very high light-scattering molecular weights to insufficient optical clarification, Boedtker and Doty carried out a rigorous examination of various samples of collagen solutions at different concentrations and after different times of ultracentrifugation. Their results showed that a stepwise removal of a high scattering component took place with an increase in time and speed of centrifugation, the light-scattering molecular weight decreasing also stepwise to the minimum value of 340,000. If the value of parent gelatin was 70,000 (Gallop) the collagen molecule would probably be made up of 5 basic units of the former material. Boedtker and Doty found, however, the

light-scattering molecular weight of the parent gelatin to be 135,000 (confirmed by osmometry and sedimentation-viscosity) after complete denaturation giving a ratio of collagen : parent gelatin of 2·5 : 1, indicating that the molecules of parent gelatin must be of substantially different weights. It is likely that the discrepancy between the light-scattering molecular weights of 70,000 (Gallop) and 135,000 (Boedtker and Doty) for parent gelatin was caused by hydrolysis. Boedtker and Doty found that the thermal and ageing effects of this process were rather critical. Possible structures of the collagen molecule interpreted from their measurements and those of X-ray studies have been suggested.

Haemoglobin

The physiological importance of haemoglobin and the ease with which it can be prepared in a pure crystalline form has led to its extensive study by various physical methods. The first accurate value for the molecular weight of haemoglobin (67,000) was reported by Adair[68] in 1925 in his investigations on the osmotic pressure of solutions of haemoglobin from different sources. At that time analysis of the iron content gave a precise estimate of the minimum molecular weight (16,700, giving a molecular weight of $4 \times 16{,}700 = 66{,}800$) and this fact made haemoglobin a useful model in the kinetics and thermodynamics of protein solutions. Subsequent work has elucidated the shape and extent of hydration of haemoglobin and also the structural and other changes which take place under different conditions (see Gutfreund[69]). A recent survey of the properties and genetic control of human haemoglobin is given by Itano[70].

By suitable adjustment of conditions, the properties of mammalian haemoglobin indicated that the molecule consisted of four sub-units. Reichman and Colvin[71] tested this supposition by light-scattering experiments on horse haemoglobin. The results showed that in dilute salt solutions below pH 2·5 the haemoglobin dissociated into four units of approximately equal molecular weight; these were subsequently separated by electrophoresis into two different components. In the crystalline form, X-ray analysis showed[69] that the molecule consists of two identical asymmetric halves related by the dyad or two-fold rotational axis of symmetry. The light-scattering weight average molecular weight of horse haemoglobin in 0·05M sodium chloride and pH 2 was 21,000 to 23,000 (osmotic pressure gave 16,000 and equilibrium sedimentation 22,000) rising to 38,000 on increasing the pH to 2·65. At this pH, performic acid oxidation of horse-globin slightly increased the molecular weight to 41,000 (osmotic pressure gave 21,000). Decreasing the pH to the original value of 2·0 did not decrease the molecular weight.

Further light-scattering studies by Haug and Smith[72] on the two electrokinetically different components of horse haemoglobin showed that the faster component with molecular weight of 25,000 associated readily as the ionic strength and pH were increased. The slower component with molecular weight of 17,000 showed little tendency to associate. Evidence from the unfractionated globin indicated that the two components interacted with each other.

Determinations of the dimensions of the haemoglobin molecule have been complicated by the extent of hydration. Bragg and Perutz[73] derived an ellipsoidal shape with dimensions 71 × 53 × 53 Å for horse haemoglobin by X-ray diffraction but the observations Crick[74] suggest that the molecule is probably more irregular than an ellipsoid.

[Other important biological substances and substances having physiological activity will be discussed in Part II of this review which will appear in the April issue of the Journal. Macromolecules of more general interest will also be briefly discussed.]

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