this work. The work was financed by the National Foundation for Infantile Paralysis, and by the Agricultural Research Council.

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Bovine Serum Albumin and its Behaviour in Acid Solution

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In a recent communication Weber (1953*a*) suggested that the bovine serum albumin molecule dissociated at pH 1·86 into two sub-units of approximately equal molecular weight. This conclusion, although supported by other data, was based largely on measurements of the depolarization of fluorescence from solutions of protein-fluorescent dye conjugates, but it was strongly contested, particularly by Pedersen (1953). Since other workers, using light scattering (Doty & Steiner, 1952; Edsall, Edelhoch, Lontie & Morrison, 1950) or osmotic pressure (Gutfreund, 1954) have unequivocally demonstrated the constancy of the molecular weight from neutrality down to pH 3·3, it remains to investigate the pH range 3·3–1·8. In the present communication, a detailed examination by light-scattering and sedimentation velocity under these conditions has been undertaken, which with published data from other workers (Champagne & Sadron, 1954) shows clearly that no significant change in molecular weight occurs. [During the preparation of this manuscript, the paper by Reichmann & Charlwood (1954) appeared, in which, on the basis of light-scattering and sedimentation measurements, the same conclusion was reached.] A parallel investigation by depolarization of fluorescence, with other optical measurements necessary for the detailed interpretation of results, was also carried out.

As will be shown, the changes in the depolarization of fluorescence observed by Weber at acid pH values have, to a large extent, been confirmed and, in view of the constancy of the molecular weight of the protein, an explanation is of some importance. In particular, a decision as to whether the changes are due to definite internal changes (involving intramolecular rotations) in the protein molecule or to alteration in the fluorescence characteristics of the conjugating molecule is required. As to the latter possibility, the dimethylamino group of the fluorescent label (1-dimethylaminonaphthalene-5-sulphonamido) used by Weber and in some of the present work has a pK value of about 4, and it is well known (Bowen & Wokes, 1953; Förster, 1951) that ionization may affect fluorescence behaviour. Accordingly, conjugates of bovine serum albumin with β-anthryl *iso*cyanate, a fluorescent non-ionizing compound used by Creech & Jones (1941), have been prepared and compared in behaviour with the naphthyl conjugates. In order to detect changes in fluorescence yield (which is related to the lifetime of the excited state), absorption and fluorescence spectra have been

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compared. It will be shown that, only if such auxiliary measurements can be made, is it feasible to apply the depolarization method over a range of experimental conditions, or in the investigation of an unknown macromolecule. For more complicated and reacting systems (Tsao, 1953), its application is much more hazardous.

EXPERIMENTAL

'Armour' crystallized bovine serum albumin was used throughout this work. Concentrations were determined after dialysis against the solvent, by measuring interferometrically the refractive index difference Δn between solution and solvent. A value of 0·1883 was taken for dn/dc (Perlmann & Longsworth, 1948; Halwer, Nutting & Brice, 1951). Measurements of pH were made in a standard Cambridge pH meter, using a glass and calomel electrode assembly which was calibrated by 0·05M potassium hydrogen phthalate at pH 3·97.

Preparation of conjugates

Preparation from 1-dimethylaminonaphthalene-5-sulphonyl chloride ('naphthyl' conjugates).

The protein solution of concentration approx. 1%, in 1% $NaHCO_3$ solution, was allowed to react with 1–2% of its weight of 1-dimethylaminonaphthalene-5-sulphonyl chloride, dissolved in a small volume of A.R. acetone, in the manner described by Weber (1952*a*). The resultant conjugate solutions, after dialysis against 0·2M-KCl to remove the main bulk of the free and adsorbed dyestuff, were then passed through an equilibrated column of basic ion-exchange resin (Dowex 2, mesh 200) to remove remaining traces. Conjugates were then tested for the absence of adsorbed material by an ascending paper chromatogram (Weber, 1953*a*). The absorption maximum of the conjugating group at about 3300 Å is sufficiently far removed from the protein absorption band to be unaffected by it; hence the number of conjugating groupings per molecule of protein was determined from the absorption at this wavelength, using for the molar extinction coefficient ϵ the value $4{\cdot}3 \times 10^6$ cm.²/mole (Weber, 1952*a*).

Preparation of conjugates from β-anthryl isocyanate ('anthryl' conjugates). These were prepared from β-anthryl *iso*cyanate (solution in A.R. dioxan) and bovine serum albumin (in 0·1M-Na_2HPO_4), in the manner described by Creech & Jones (1941) for the conjugation of horse serum albumin. However, since only a lightly labelled conjugate was required (approx. 3 mol./mol. of protein), the amount of *iso*cyanate employed was reduced to about 20 mg./g. of protein. The resultant anthryl conjugates were freed of extraneous *iso*cyanate by two precipitations with 2·8M-$(NH_4)_2SO_4$, followed by a final precipitation with acetone at −18°. The degree of conjugation was calculated from the absorption maximum of the β-anthryl carbamido-group which occurred at 3550 Å, using a value of ϵ of $3{\cdot}04 \times 10^6$ cm.²/mole, calculated from the value of $E^{1\%}_{1\,cm.} = 139$, given by Creech & Jones.

Depolarization of fluorescence measurements

Measurement of the degree of polarization of fluorescent radiation, over a temperature range 4–40°, was made in an apparatus similar to that described by Perrin (1929) and Weber (1952*b*), fluorescence being excited by means of a mercury arc and a Wood's glass filter. Observations of fluorescence depolarization were made at right angles to the direction of excitation, through an Ilford filter no. 110. We have found, however, that this polariscope, employing the method of observing the disappearance of fringes, was subjective and lacking in sensitivity. We have, therefore, adapted a polariscope of the type described by Wright (1934), which employs matching of fringes, a more sensitive and impartial procedure (W. F. Harrington, P. Johnson & R. H. Ottewill, unpublished).

It has been shown by Weber (1952*b*) that depolarization of the fluorescent radiation from conjugated proteins which are oblate or prolate ellipsoids of small asymmetry obeys a law analogous to that of Perrin (1926) for spherical molecules. For unpolarized exciting radiation, the equation may be written

$$\frac{1}{p}+\frac{1}{3}=\left(\frac{1}{p_0}+\frac{1}{3}\right)\left(1+3\frac{\tau_0}{\rho_h}\right), \qquad (1)$$

where p represents the degree of polarization of fluorescent light emitted at right angles to the direction of the incident radiation, τ_0 the lifetime of the excited state of the fluorescence, ρ_h the harmonic mean of the two principal relaxation times of the rotation of the ellipsoidal molecule, and p_0 is an empirical constant. The value of p_0 may be determined experimentally by measuring the depolarization of the fluorescence of the conjugate in 60% (w/v) sucrose solution.

If the relation between $1/p$ and T/η is linear (T is the absolute temperature and η the viscosity of the solvent) and b is the slope of this curve, then, provided that the value of τ_0 is not dependent on temperature, a quantity β may be defined, where

$$\beta=\frac{b}{(1/p_0+\frac{1}{3})}=\frac{3\eta\tau_0}{\rho_h T}. \qquad (2)$$

The determination of ρ_h, over a range of temperature, requires ideally a comparable knowledge of the absolute value of τ_0 for the conjugated protein, determined by a technique of the type described by Bailey & Rollefson (1953). This, however, is a problem of some magnitude and, of the other more approximate methods available, that devised by Lewis & Kasha (1945), or in an improved form by Förster (1951), may be employed. Thus τ_0 can be derived from Förster's equation:

$$\frac{1}{\tau_0}=2{\cdot}88\times10^{-9}\,\frac{n^2\nu^2_{max.}}{Q}\int_0^\infty \epsilon_\nu d\nu \text{ sec.}^{-1}, \qquad (3)$$

where n represents the refractive index of the medium, $\nu_{max.}$ the maximum of the absorption band in wave numbers, and $\int_0^\infty \epsilon_\nu d\nu\,(=A)$ the area of the absorption band. The fluorescence yield, Q, is defined as

$$\frac{\text{No. of fluorescent quanta emitted}}{\text{No. of incident quanta absorbed}}=\frac{kF}{A},$$

where k is a constant and F and A are areas under the fluorescence and absorption bands respectively. Hence equation (3) may be written

$$\frac{1}{\tau_0}=\frac{K\nu^2_{max.}A^2}{F}, \qquad (4)$$

where K is a constant. It follows that

$$\frac{\tau}{\tau_0}=\frac{\nu^2_{0\max.}A_0^2F}{\nu^2_{\max.}A^2F_0}. \quad (5)$$

Thus if the value of τ_0 can be well established in one environment, it is possible to estimate the value of τ in any other by a direct comparison of the absorption and fluorescence spectra in the two environments, measured under otherwise identical conditions of protein concentration and labelling.

Simpler cases result if (1) there is little or no shift in the absorption maximum between the two environments, giving

$$\frac{\tau}{\tau_0}=\frac{A_0^2F}{A^2F_0}, \quad (6)$$

and (2) the absorption characteristics are completely unchanged in both environments, when

$$\frac{\tau}{\tau_0}=\frac{F}{F_0}. \quad (7)$$

By accepting Weber's value of τ_0 under neutral conditions (Weber, 1952*a*), the value of τ at acid pH may be obtained by a comparison of the fluorescence and absorption spectra (at concentrations below the range of concentration quenching) at neutral and acid pH. The value of ρ_h may then be corrected by equation (2).

If, alternatively, measurements of the depolarization of the fluorescence of the conjugating molecule in a viscous solvent, e.g. glycerol, are substituted in the Perrin equation for unpolarized exciting radiation:

$$\frac{1}{p}+\frac{1}{3}=\left(\frac{1}{p_0}+\frac{1}{3}\right)\left(1+\frac{RT}{\eta V}\tau_0\right), \quad (8)$$

then τ_0 may be obtained if some estimate can be made of the solvated volume V of the fluorescent molecule in solution. This is a difficult estimation, and moreover the value of τ_0 obtained is not necessarily that of the conjugating grouping when it is attached to the protein. Weber calculated the value of ρ_h for bovine serum albumin and ovalbumin at the isoelectric points from the dielectric dispersion measurements of Oncley (1942), and, combining this with depolarization of fluorescence measurements, he found for naphthyl conjugates $\tau_0=1{\cdot}4\times10^{-8}$ sec. By employing this procedure for anthryl conjugates, a value of $\tau_0=4{\cdot}4\times10^{-8}$ sec. is obtained. Substitution in the Perrin equation gives $V=398$ ml., for β-anthryl *iso*cyanate in glycerol, a value which is in excess of the molecular volume in the solid state by a factor which is of the same order as for several other fluorescent substances studied by Marinesco (1927). The value of τ_0 is of the same order as that for anthracene in acetone, 5×10^{-8} sec., obtained by Rau (1949), using a fluorometer.

Absorption spectra and fluorimetry

Absorption spectra were measured by a Unicam photoelectric quartz spectrophotometer type SP. 500.

Fluorescent intensity curves were obtained by using the fluorimeter attachment supplied for use with this instrument. The fluorescent standard employed was a solution of $1{\cdot}5\times10^{-5}$ g./ml. of 1-dimethylaminonaphthalene-5-sulphonic acid in dilute aqueous $NaHCO_3$. All solutions were filtered, before measurement, through a sintered-glass disk.

Light-scattering

The measurements were carried out with the light-scattering apparatus described by Goring & Johnson (1952*a*), and, as incident light, the mercury green line 5461 Å was used. Solutions were clarified by ultrafiltration through collodion membranes prepared according to the directions of Goring & Johnson (1952*b*). Weight-average molecular weights, M, were calculated from the usual light-scattering equation for small symmetrical scatterers:

$$\frac{Hc}{\tau}=\frac{1}{M}+\frac{2bc}{RT}=\frac{1}{M}+2Bc, \quad (9)$$

where c = concentration of solution (g./ml.), H contains optical terms and b ($=RTB$) is a constant from the osmotic pressure–concentration equation. For Rayleigh scatterers the turbidity τ is proportional to the scattering I_{90},

$$\tau=CI_{90}, \quad (10)$$

where C is a calibration factor, and for the apparatus used had the value $4{\cdot}35\times10^{-3}$ cm.$^{-1}$. All experiments were carried out at room temperature, $18\pm2°$.

Fluorescence spectra

Since no specific apparatus for detailed investigation of fluorescence spectra was available, the light-scattering apparatus was modified for this purpose. Solutions were placed in the normal light-scattering cells, and fluorescence was excited by means of a mercury arc and a Wood's glass filter. By the use of a series of interference filters, with transmissions in the range 4000–6000 Å, in front of the electron multiplier, the intensity of fluorescence in the 90° position as a function of wavelength was investigated. The resultant spectra were then corrected for the spectral sensitivity of the photomultiplier and the transmission factor of the filters. Fluorescence intensities obtained in this manner have to be corrected for the polarization of the fluorescent emission, the appropriate equation being that given by Singleterry & Weinberger (1951):

$$I_{\text{av.}}=I_{90}\left(\frac{6-2p}{6-3p}\right), \quad (11)$$

where $I_{\text{av.}}$ is the intensity that would have been observed with complete depolarization of the emitted light. Solutions of the conjugated proteins were thus compared at neutral and acid pH but under otherwise identical conditions of protein and labelling dye concentration. In order to utilize equation 3, the product $I_{\text{av.}}\nu$ was plotted against ν, giving what are henceforth termed modified fluorescence spectra.

The resolution obtained by this method is not high, but since no significant spectral displacements were involved, it was thought to be sufficiently good to indicate gross changes, and to be preferable to the photographic method with its multiplicity of corrections. However, for a more quantitative investigation of life-time changes, a more detailed study of the fluorescence spectra would be required.

Sedimentation

Sedimentation-velocity experiments were carried out with a Phywe air-driven ultracentrifuge equipped with a Philpot diagonal schlieren optical system. The temperature rise of the rotor was recorded throughout each run by means of a thermistor placed 1 mm. above the rotor surface

some 3 cm. from the axis of rotation (P. Johnson, unpublished work). A correction factor was computed to reduce sedimentation constants to the viscosity and density of water at 20°.

Optical rotation

Optical-rotation measurements were made in a modified Schmidt & Haensch polarimeter. The polarimeter tube was maintained at the desired temperature (±0·2°) by pumping water from a thermostatically controlled bath through the outer jacket.

RESULTS

Naphthyl conjugates

Depolarization of fluorescence measurements have been carried out on naphthyl conjugates of bovine serum albumin in phosphate buffer (pH 7·3, *I* 0·05), in distilled water adjusted to pH 1·9 with hydrochloric acid, in sodium chloride solutions of different ionic strengths at a pH of 1·9, and in 7 M urea solution (Fig. 1). In the latter case, in order to obtain a sufficient range of T/η values, it was necessary to examine the depolarization of fluorescence at constant temperature (18°) in 7 M urea solutions containing varying proportions of sucrose. The dynamic viscosity of each solution was then determined from the relative kinematic viscosity and the density. To avoid quenching effects as far as possible, low degrees of labelling (less than 5 groups/protein molecule) and low protein concentrations (less than 0·1 g./100 ml.) were used, but within the range examined depolarization appeared to be independent of protein concentration.

The values of p_0 obtained by extrapolation with the solutions at pH 7·3, and in dilute hydrochloric acid at pH 1·9, were in good agreement with the values obtained by measurements of depolarization of fluorescence in 60% (w/v) sucrose. The plots in sodium chloride solutions are, however, slightly convex to the T/η axis, an indication of an increasing degree of rotation with temperature. Assuming the value of T/η for water at 25° to be $3{\cdot}33 \times 10^4$ (degrees/poise), and τ_0 to be $1{\cdot}4 \times 10^{-8}$ sec., the ρ_h values given in Table 1 were calculated. In the remainder of this paper, it will be assumed that ρ_h values refer to water at 25°. Where the plots show curvature, the slopes towards their extremities are used in deducing the corresponding ρ_h values.

The absorption spectra of the conjugating group on the protein at neutral and acid pH were determined by subtraction of the protein contribution (Fig. 2). It was found that no gross change in the absorption spectra of bovine serum albumin between pH 1·8 and neutrality occurs. With the fluorescence spectra (Fig. 3) no correction was necessary for the contribution of the protein, since the amount of scattering at the protein concentration employed (0·035%) was negligible compared with the fluorescence intensity. A small correction, by equation (11), was applied for the

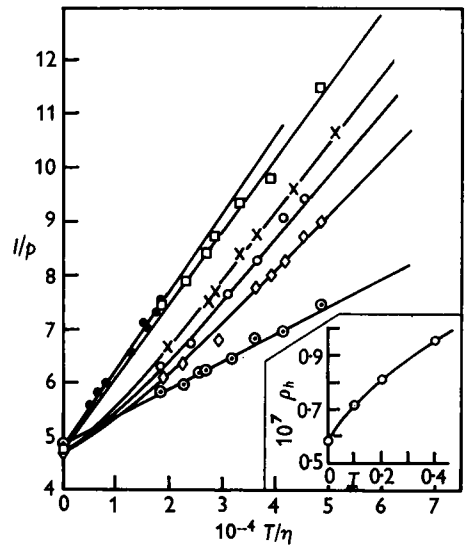

Fig. 1. Fluorescence polarization of naphthyl conjugates of bovine serum albumin (2·7 mol. of naphthyl derivative/mol. of protein), $1/p$ versus T/η curves in: ●, 7 M urea solution; □, dilute hydrochloric acid, pH = 1·9; ×, dilute hydrochloric acid + 0·1 M-NaCl, pH = 1·9; ○, dilute hydrochloric acid + 0·2 M-NaCl, pH = 1·9; ◇, dilute hydrochloric acid + 0·4 M-NaCl, pH = 1·9; ⊙, phosphate buffer, pH 7·3, *I* 0·05. Protein concentrations of solutions, 0·02–0·08 g./100 ml.

Inset. Rotational relaxation time ρ_h (corrected) at low T/η values versus ionic strength.

Table 1. *Variation of rotational relaxation time* ρ_h (25°) *with solvent for naphthyl conjugates (assuming* $\tau_0 = 1{\cdot}4 \times 10^{-8}$ *sec.)*

Solution	pH	$1/p_0$	$10^7 \rho_h$ (sec.) (±2%) High T/η	Low T/η
Dilute HCl	1·90	4·76±0·05	0·47	0·47
0·1 M-NaCl + HCl	1·97	4·59	0·47	0·58
0·2 M-NaCl + HCl	1·92	4·60	0·46	0·66
0·4 M-NaCl + HCl	1·92	4·60	0·59	0·78
Phosphate buffer	7·3	4·83	1·28	1·28
7 M urea	—	4·80	0·45	0·45

polarization of the fluorescence. From the integrated areas of the absorption and emission curves, the values of τ in the different environments were calculated and the values of ρ_h corrected accordingly (Table 2).

The value of $1{\cdot}28 \times 10^{-7}$ sec. for bovine serum albumin at pH 7·3, I 0·05, is in good agreement with $1{\cdot}27 \times 10^{-7}$ sec. found experimentally by Weber (1952*a*), and $1{\cdot}24 \times 10^{-7}$ sec. calculated from the dielectric-dispersion measurements of Oncley (1942). The uncorrected value of $0{\cdot}47 \times 10^{-7}$ sec. found in dilute HCl at pH 1·9 is lower than Weber's (1953*a*) value (also uncorrected) of $0{\cdot}56 \times 10^{-7}$ sec., found under these conditions. After correction for life-time changes these values become $0{\cdot}58 \times 10^{-7}$ and $0{\cdot}69 \times 10^{-7}$ sec. respectively. On the addition of NaCl at pH 1·9 the rotational relaxation times at low T/η values increase (Fig. 1, inset), but at higher T/η values they approach $0{\cdot}58 \times 10^{-7}$ sec., except in 0·4 M-NaCl. In the latter calculations, the intercept on the $1/p$ axis of the linear plot at high T/η values was used as the $1/p_0$ value. Thus though the changes in the lifetime of the excited state modify the apparent relaxation times in acid solution, they cannot in themselves wholly account for the reduction observed at pH 1·9. It is also of interest that in 7 M urea the ρ_h value for bovine serum albumin is still further reduced to $0{\cdot}37 \times 10^{-7}$ sec., indicating slightly greater rotational freedom than in dilute HCl at pH 1·9.

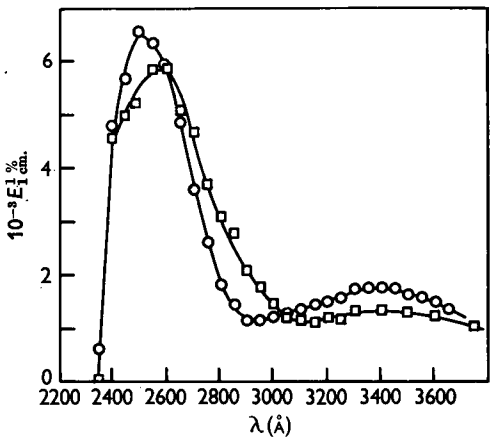

Fig. 2. Absorption spectra of the naphthyl grouping conjugated to the bovine serum albumin molecule (2·7 mol. of naphthyl derivative/mol. of protein) at: ○, pH 5·6; □, pH 2·0.

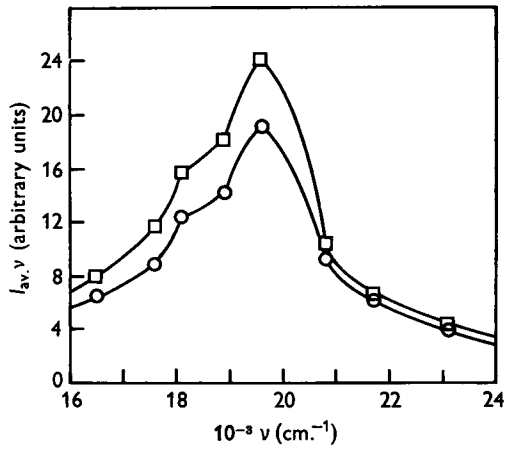

Fig. 3. Modified fluorescence spectra of naphthyl conjugates of bovine serum albumin (2·7 mol. of naphthyl derivative/mol. of protein) at: ○, pH = 5·6; □, pH = 2·0. Protein concentration, 0·035 g./100 ml.

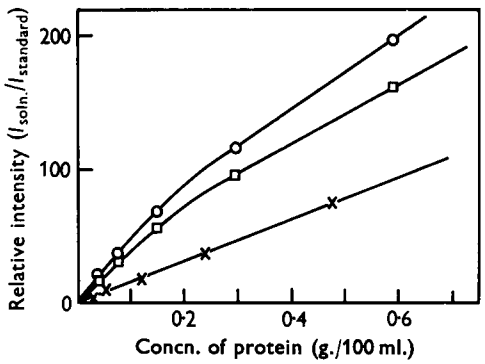

Fig. 4. Fluorescent intensity–protein concentration for naphthyl conjugates (4·9 mol. of naphthyl derivative/mol. of protein) in: ○, dilute hydrochloric acid at pH 1·9; □, distilled water at pH 5·3; and ×, 7 M urea.

Table 2. *Values of rotational relaxation time ρ_h corrected for variation in the life time of the excited state (naphthyl conjugates)*

Solution	pH	$10^8\ \tau$ (sec.)	$10^7\ \rho_h$ (sec.) (±2%) High T/η	Low T/η
Dilute HCl	1·90	1·72	0·58	0·58
0·1 M-NaCl + HCl	1·97	1·72	0·58	0·71
0·2 M-NaCl + HCl	1·92	1·72	0·57	0·81
0·4 M-NaCl + HCl	1·92	1·72	0·73	0·96
Phosphate buffer	7·3	1·40	1·28	1·28
7 M urea	—	1·15	0·37	0·37

In Fig. 4 are shown the curves of relative total fluorescence intensity versus concentration of protein over a wide range of protein concentration for a sample containing 4·9 mol. of naphthyl derivative/mol. of protein. These demonstrate clearly the increase in fluorescent intensity that occurs with the naphthyl conjugate at pH 1·9 (cf. Fig. 3) in comparison with that under neutral conditions. With the free naphthylsulphonic acid, quenching is observed in the neighbourhood of pH 4. Weber (1953*b*) has attributed the lack of quenching of the conjugated protein to the strongly charged environment of the protein molecule. The quenching effect caused by 7 M urea solutions is also clearly shown in Fig. 4.

Anthryl conjugates

Studies on the depolarization of fluorescence have been carried out on anthryl conjugates, in phosphate buffer (pH 7·3, I 0·05), and in dilute hydrochloric acid at pH 1·9. The curves obtained with these conjugates obey the linear law of depolarization (Fig. 5), but the polarization values obtained are smaller than those observed with naphthyl conjugates owing to the longer lifetime of the excited state ($4{\cdot}4 \times 10^{-8}$ sec. at pH 7·3). The values of ρ_h obtained at pH 7·3 and 1·9 are 1·24 and $0{\cdot}80 \times 10^{-7}$ sec. respectively.

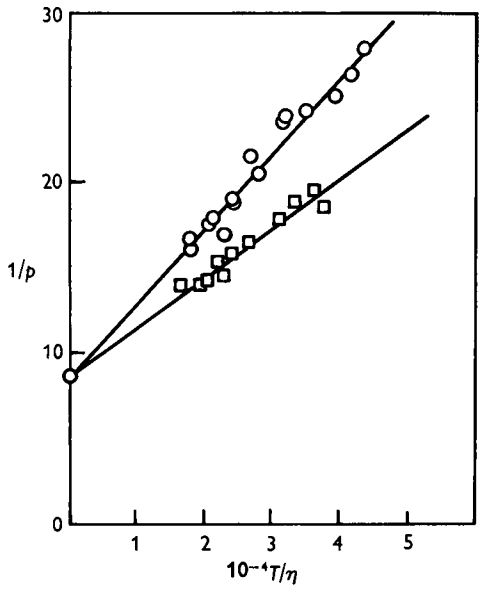

Fig. 5. Fluorescence polarization of anthryl conjugates of bovine serum albumin (3·9 mol. of anthryl derivative/mol. of protein), $1/p$ versus T/η curves in: ○, dilute hydrochloric acid at pH 1·9; □, phosphate buffer, pH 7·3, I 0·05. Protein concentrations approx. 0·02 g./100 ml.

To investigate possible changes in the lifetime of the excited state, the absorption and fluorescence spectra have been examined (Figs. 6, 7). A considerable increase in fluorescence intensity occurred at pH 1·9; this was confirmed by means of fluorimetric curves (Fig. 8), which also indicated that, in order to obtain data on the polarization of fluorescence on the linear portion of these curves, concentrations of about 0·02 % of conjugate had to be employed. At the same time, however, an increase

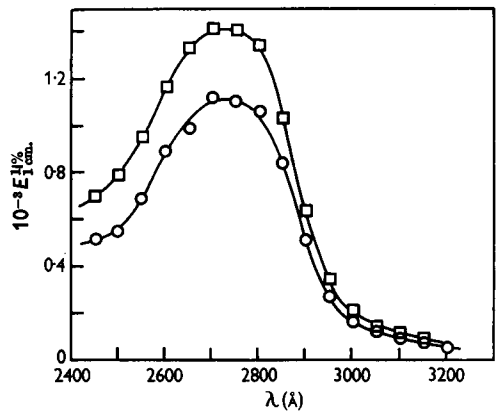

Fig. 6. Absorption spectra of the anthryl grouping conjugated to the bovine serum albumin molecule (3·9 mol. of anthryl derivative/mol. of protein). ○, pH 7·3, I 0·05; □, pH 1·9.

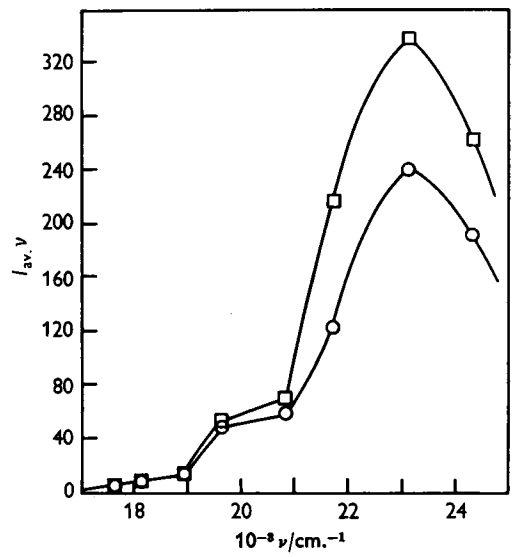

Fig. 7. Modified fluorescence spectra of anthryl conjugates of bovine serum albumin (3·9 mol. of anthryl derivative/mol. of protein) at: ○, pH 7·3, I 0·05; □, pH 1·9. Protein concentration, 0·02 g./100 ml.

in absorption was observed, and, from the integrated areas of the fluorescent and absorption spectra, the lifetime of the excited state was calculated to be $3{\cdot}72 \times 10^{-8}$ sec. at pH 1·9, giving a corrected ρ_h value of $0{\cdot}68 \times 10^{-7}$ sec.

Although somewhat different from the value of $0{\cdot}59 \times 10^{-7}$ sec. obtained for the naphthyl conjugates (compare the corrected value of Weber, $0{\cdot}69 \times 10^{-7}$ sec.), a difference which may possibly be accounted for by errors in the lifetime corrections, it is appreciably different from the value of $1{\cdot}28 \times 10^{-7}$ sec. obtained at neutral pH. It appears, therefore, that the major effects observed in bovine serum albumin conjugates (naphthyl or anthryl) by depolarization of fluorescence are not a function of the labelling group, and must to an appreciable extent be related to changes occurring in the protein molecule.

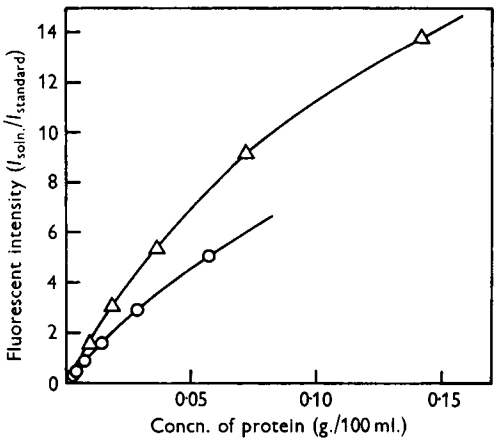

Fig. 8. Fluorescent intensity–protein concentration for anthryl conjugates (3·9 mol. of anthryl derivative/mol. of protein) in: △, dilute hydrochloric acid at pH 2·0; □, distilled water at pH 5·3.

Light-scattering

Light-scattering measurements have been carried out on solutions of bovine serum albumin at pH values of 4·5, 3·5, 2·3 and 1·86; the constitution of the solutions was as recorded in Table 3. Within experimental error all the curves gave good linear extrapolations to $(Hc/\tau)_{c=0} = 1{\cdot}39 \times 10^{-5}$ (Fig. 9), which gives a weight-average molecular weight of 72 000 ± 2000. The values of the gradient ($2B$), and the dissymmetries (I_{60}/I_{120}) of the most concentrated solutions at each ionic strength are recorded in Table 3. As stated elsewhere (Goring & Johnson, 1952*a*), variations in the dissymmetries, such as are observed, are not considered significant.

No aggregation effects of the type noticed by Goring (1952) were found in this work. It seems likely that the effects observed by him may have been due to a fatty acid impurity, occurring in some samples of bovine serum albumin, which is precipitated at acid pH (Yang & Foster, 1954). However, since the solutions in the present work were always ultrafiltered after adjustment of pH, it is unlikely that any effect of this type would have been encountered. It may therefore be concluded that no change of molecular weight occurs with bovine serum albumin in the pH range 4·5–1·9, at I 0·10.

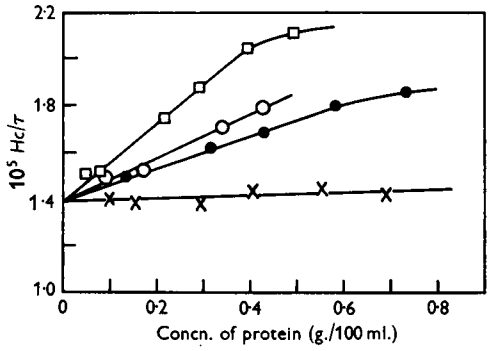

Fig. 9. Light-scattering curves of Hc/τ versus c for bovine serum albumin in the pH range 1·86–4·5. □, pH 2·3, I 0·065; ○, pH 1·86, I 0·1; ●, pH 3·5, I 0·1; ×, pH 4·5, I 0·1.

Optical rotation

The optical rotation of solutions of bovine serum albumin in water and 0·1 M-NaCl to which sufficient HCl was added to give pH 1·9 was measured as a function of wavelength and temperature (Fig. 10). Linderstrøm-Lang & Schellman (1954) have shown that the wavelength variation may be expressed in the form

$$\frac{1}{[\alpha]} = \frac{\lambda^2}{A} - \frac{\lambda_c^2}{A}, \qquad (12)$$

Table 3. *Light-scattering functions for bovine serum albumin at acid* pH

Solution	pH	Ionic strength	Dissymmetry (I_{60}/I_{120})	$2B \times 10^5$ (eqn. 9)
HOAc + NaOAc + NaCl	4·5	0·10	1·05	5·7
HOAc + NaOAc + NaCl	3·5	0·10	1·05	69·9
HCl + glycine + NaCl	2·3	0·07	1·02	165
HCl + 0·1 M-NaCl	1·9	0·11	1·09	93·9

where A and λ_c are empirical constants for a protein under given conditions; λ_c seems to be a measure of the amount of secondary structure occurring in the protein. For native proteins it has a value 2450–2650 Å and for denatured materials it covers the range 2000–2300 Å. As Fig. 10 (*inset*) shows, $\lambda_c = 2650$ Å for bovine serum albumin at pH 1·9, somewhat larger than the value (2550 Å) given for the neutral protein. The occurrence of

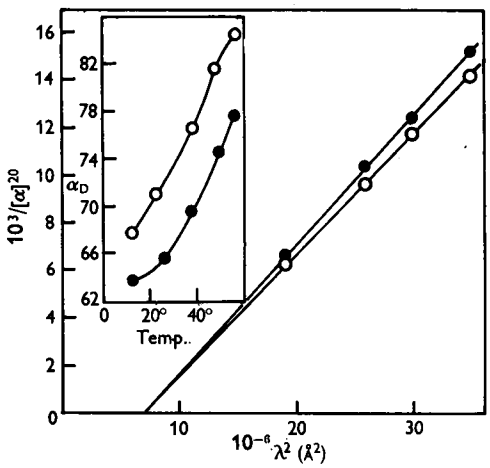

Fig. 10. Optical rotation measurements: $1/[\alpha]^{20}$ versus λ^2 for bovine serum albumin at pH 1·9 and protein concentration of 6·7 g./100 ml. ○, *I* 0; ●, *I* 0·1.
Inset. $[\alpha]_D$ versus temperature.

positive temperature coefficients of optical rotation has been found by Schellman (1955) to be associated with proteins having largely intact secondary structure, whereas denatured proteins invariably give a zero or negative temperature coefficient.

Sedimentation velocity

The sedimentation-velocity method was also used to investigate the effect of changes in ionic strength and pH on the molecule of bovine serum albumin. As a control the sedimentation coefficient as a function of protein concentration in 0·1 M-NaCl at pH 5·1 was determined. Fig. 11 summarizes these results and contains also the results of Creeth (1952) and of Kegeles & Gutter (1951), corrected in each case for the adiabatic cooling effect noted by Waugh & Yphantis (1952) and now accepted by many workers, e.g. Biancheria & Kegeles (1954). In each case a cooling of 1°, appropriate to a rotational speed of 60 000 rev./min., was assumed. A considerable measure of agreement between the different groups of results is apparent. The least-squares line for only the present results is given by the full line of Fig. 11, and its equation is

$$S^0_{20}\text{ (Svedberg units)} = 4{\cdot}51(\pm 0{\cdot}05) - 0{\cdot}32(\pm 0{\cdot}11)\,c. \quad (13)$$

Standard errors are given in parentheses. In evaluating their results, Kegeles & Gutter (1951) ignored sedimentation data at concentrations of 0·2 g./100 ml. and below on the grounds that, under such conditions, heavy impurities caused a spurious elevation of the sedimentation coefficient. In the present work no trend in the sedimentation

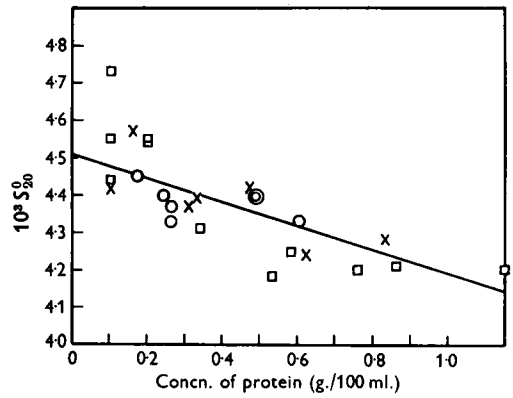

Fig. 11. Sedimentation coefficient S^0_{20} versus protein concentration for bovine serum albumin. ×, Present work; □, Kegeles & Gutter (1951); ○, Creeth (1952).

coefficient during long runs was observed and all the results were used in calculating the constants of equation (13). Creeth, who also utilized data below 0·2 g./100 ml. concentration, obtained

$$S^0_{20} = 4{\cdot}42(\pm 0{\cdot}03) - 0{\cdot}12(\pm 0{\cdot}08)\,c \quad (14)$$

to be compared with

$$S^0_{20} = 4{\cdot}38 - 0{\cdot}069c \quad (15)$$

by Kegeles & Gutter. Although the s–c slopes of the Spinco investigations are by no means in quantitative agreement, they are considerably smaller than that obtained in the present work, which is similar to that observed by Koenig & Pedersen (1950) over a wide concentration (up to 5% of protein) range. Sedimentation coefficients in the latter work are consistently at a higher level than those already quoted, but this would not be expected to affect the slope of the regression line. It is clear that the value for the slope of the S^0_{20}–c line must remain uncertain until more precise results are available. For the sedimentation coefficient extrapolated to zero concentration $[S^0_{20}]$, the possible ranges of values given by the different recent workers are closely adjacent, and a rounded value of $[S^0_{20}] = 4{\cdot}45$S is to be recommended. It

seems highly unlikely, from the different experimental approaches supporting this value, that it can be in error by more than 0·1S.

To investigate the variation of sedimentation coefficient with pH at ionic strength, I 0·1, a constant protein concentration of 0·32 g./100 ml. was used; at this concentration well-defined boundary peaks and sedimentation coefficients approaching those at infinite dilution are obtained. Fig. 12 summarizes the results obtained (with other physical measurements) and includes data from Svedberg & Sjögren (1930) obtained at 0·25% protein concentration. The fall in sedimentation coefficient as the pH is lowered below 4 is clearly shown by both sets of data, and, at a pH of about 2, the curve appears to flatten out at a value 3·43S.

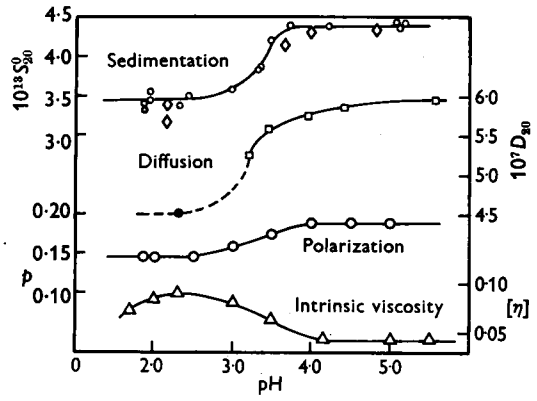

Fig. 12. Variation of sedimentation coefficient (○, I 0·1, present work; ◇, I 0·02–0·1, Svedberg & Sjögren, 1930), diffusion coefficient (□, I 0·15, Champagne & Sadron, 1954; ●, I 0·33, Kekwick, personal communication), polarization of fluorescent conjugates at I 0·02 and 20° (Weber, 1952*a*), and intrinsic viscosity at I 0·1 (Yang & Foster, 1954), with pH for bovine serum albumin.

The effect of changing ionic strength on sedimentation coefficient was investigated at constant protein concentration (0·32 g./100 ml.) and at two different levels of pH. Although at the higher pH (5·1) changes in ionic strength over the range 0·1–0·5 had no effect, at pH 1·90 pronounced effects were observed (Fig. 13). Thus below I 0·3, reduction in ionic strength was accompanied by lowered sedimentation coefficient, an extrapolated value of 3·20S, corresponding to zero ionic strength, being obtained. Between I 0·3 and 0·6 the sedimentation coefficient, 3·75S, was apparently independent of ionic strength. Above I 0·6, some precipitation as well as aggregation of the protein occurred, demonstrated by the sedimentation diagrams of Fig. 14. It is to be noted that sedimentation coefficients of naphthyl and anthryl conjugates of bovine serum albumin fell, within experimental error, upon the curves for the unconjugated protein (Fig. 13), so that the presence of the conjugating agent does not affect the kinetic properties of the protein.

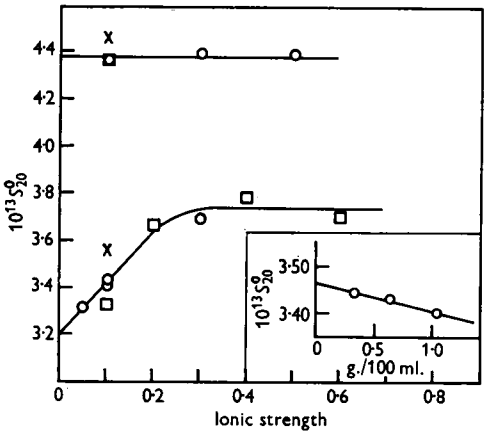

Fig. 13. Sedimentation coefficient, S^0_{20}–ionic strength for bovine serum albumin at pH 1·9 and 5·1. ○, Unconjugated bovine serum albumin; □, naphthyl conjugate; ×, anthryl conjugate. Protein concentration approx. 0·3 g./100 ml.

Inset. Sedimentation coefficient, S^0_{20} versus protein concentration for bovine serum albumin at pH 1·9, I 0·1.

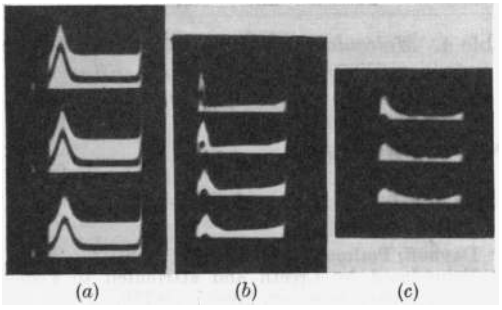

Fig. 14. Sedimentation diagrams for bovine serum albumin at pH 1·9 in different concentrations of sodium chloride. (*a*) 0·1M-NaCl, 0·62% protein; (*b*) 0·6M-NaCl, 0·32% protein; (*c*) 1·0M-NaCl, 0·32% protein.

The curves of Fig. 13 refer to a protein concentration of 0·32 g./100 ml., and since the effect of acid conditions on the slope of the S^0_{20}–c line was not known, a few runs at I 0·1, pH 1·9, were carried out at different protein concentrations. The data shown in Fig. 13 (*inset*) are not sufficient to give the S^0_{20}–c slope with accuracy, but the line drawn through the experimental results gives a slope which is quantitatively similar to that of Kegeles & Gutter under more neutral conditions. An $[S^0_{20}]$ value of 3·46S

was obtained by extrapolation, but it is less accurate than the isoelectric value. The partial specific volume determined pyknometrically at I 0·1, pH 1·90 in NaCl–HCl solution at 20° was found to be $0·729 \pm 0·005$, in good agreement with that of Koenig (1950) under neutral conditions, but somewhat lower than that of Dayhoff, Perlmann & MacInnes (1952).

DISCUSSION

Molecular weight and frictional ratio

Diffusion coefficient measurements on bovine serum albumin have recently been performed by Creeth (1952), as well as by Champagne & Sadron (1954), somewhat differing results being reported. The latter authors obtained a value

$$5·96(\pm 0·02) \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1},$$

independent of ionic strength (above I 0·05) and protein concentration in the neighbourhood of the isoelectric point, but dependent on pH. Under similar conditions, Creeth obtained the value $6·14\ (\pm 0·02) \times 10^{-7}$, which was apparently independent of protein concentration and of small changes in ionic strength and pH. By combining these with the value $[S^0_{20}] = 4·45S$ already recommended we obtain the results of Table 4. The calculated molecular weight is rather critically dependent on the partial specific volume, but it seems clear that the molecular weight of the protein is in excess of the value (65 360) suggested by Creeth who used $\bar{v} = 0·740$ as well as Spinco sedimentation coefficients uncorrected for temperature effects. The value $\bar{v} = 0·734$ is probably accurate to $\pm 0·002$, so that the molecular weight from sedimentation and diffusion may be given as $67\,500 \pm 1500$. Narrower limits await decisive information on the diffusion coefficient.

Table 4. *Molecular weight of bovine serum albumin from sedimentation velocity and diffusion*

$[S^0_{20}] = 4·45S.$

Diffusion coeff. (D_{20}) (cm.² sec.⁻¹)	$\bar{v} = 0·734$*	$\bar{v} = 0·740$†
$5·96 \times 10^{-7}$‡	68 200	69 800
$6·14 \times 10^{-7}$§	66 200	67 700

* Dayhoff, Perlmann & MacInnes (1952).
† Value used by Creeth and attributed to Pedersen (1945). The latter, however, gives $\bar{v} = 0·736$.
‡ Champagne & Sadron (1954).
§ Creeth (1952).

Recalculation of recent osmotic data (Gutfreund, 1954) also indicates a molecular weight value in good agreement with the last estimate. Thus Gutfreund used a dn/dc value of 0·192, which is higher than is now accepted for bovine serum albumin in dilute salt solutions at λ 5600 Å (utilized in interferometry). Using $dn/dc = 0·1883$ (see page 570), we now obtain the molecular weight $67\,400 \pm 1000$. Light-scattering consistently gives molecular-weight values for bovine serum albumin of 70 000 and above (Edsall *et al.* 1950; Halwer *et al.* 1951; Goring & Johnson, 1952*a*), but in view of its weight-average character it would not appear to be a suitable method for accurate evaluation of molecular weight.

Fig. 12 contains the diffusion data of Champagne & Sadron (1954) and of R. A. Kekwick (personal communication), and the effect of pH on the diffusion coefficient is clearly very similar to that on the sedimentation coefficient. The molecular weights of Table 5 have been calculated by combining the diffusion and sedimentation data and assuming $\bar{v}$ to be 0·734. The sedimentation coefficients quoted strictly refer to 0·32 % of protein, but, for molecular-weight calculation, a slope of $-0·20$ (i.e. $S = [S^0] - 0·2c$) for the s–c curve, within the range reported by various workers, has been assumed for correction purposes. The frictional ratios of Table 5 were calculated by assuming a constant value 67 500 for the molecular weight and the diffusion coefficients of column 2.

Allowing for the approximations used, it is clear that there is no change in the state of aggregation of the protein at I 0·1 down to pH 2·3. Further, though light-scattering molecular weights are

Table 5. *Molecular weight and frictional ratio of bovine serum albumin as a function of* pH

0·1 M-NaCl. pH adjusted with HCl.

pH	$10^7 D_{20}$ (cm.² sec.⁻¹)	S^0_{20} (Svedberg units)	Mol.wt.	Frictional ratio f/f_0
5·3	6·0*	4·42	68 200	1·31
4·4	5·85*	4·41	69 800	1·35
3·95	5·74*	4·39	70 800	1·37
3·45	5·58*	4·05	67 250	1·41
3·2	5·25*	3·75	66 250	1·50
2·3	4·49†	3·43	71 000	1·75
			Mean 68 900	

* From Champagne & Sadron (1954). † From Kekwick (personal communication).

somewhat higher, they confirm this conclusion and extend the pH-stability range to 1·9; no diffusion data are available at this value. Weber (1953*a*) quotes a sedimentation coefficient at pH 1·82 in 0·2 M-KCl of 2·7S (determined by Dr R. A. Kekwick) and a diffusion coefficient of $6{\cdot}85 \times 10^{-7}$. However, neither value is consistent with the more comprehensive data of Fig. 13. Dr R. A. Kekwick (personal communication) states that the sedimentation coefficient was obtained from one run only at 1% protein and that further experiments at 0·4% protein and pH 2·3 in phosphate buffer

$$(0{\cdot}124\,\text{M-}H_3PO_4 + 0{\cdot}206\,\text{M-}KH_2PO_4)$$

gave $S^0_{20} = 3{\cdot}68$S (in fair agreement with the curve of Fig. 13 at pH 1·9) and $D_{20} = 4{\cdot}49 \times 10^{-7}$. The latter values yield a molecular weight of 74 000 for the preparation used.

Although the molecular weight does not vary as the pH is reduced to 1·9, the frictional ratio increases steadily. This behaviour is clearly reflected (see Fig. 12) in the curves against pH of intrinsic viscosity (drawn from the work of Yang & Foster, 1954) and of polarization of fluorescence of naphthyl conjugates of bovine serum albumin (see below). Yang & Foster (1954) also showed that significant changes in optical rotation occurred over the same pH range. Similarly, Gutfreund & Sturtevant (1953) detected a reversible first-order heat change (of 3100 cal.) in solutions of bovine serum albumin between pH 4·5 and 3·5. The occurrence of changes in sedimentation coefficient, diffusion coefficient, intrinsic viscosity, optical rotation, polarization of fluorescence and heat content over the pH range 4·5–2 strongly suggests a common explanation, and it seems likely that the changes are connected in some way with the increasing positive charge deriving from the titration of the carboxyl groups.

Treatment in terms of the β function

To investigate the meaning of the change in f/f_0, the approach of Scheraga & Mandelkern (1953) has been explored. Alternative suggestions made by Ogston (1953) have not been used, in view of their dependence on the $1/S$–c slope. Applying in the first place to bovine serum albumin in neutral solution and using $[S^0_{20}] = 4{\cdot}45S$, $[\eta] = 0{\cdot}038$, $M = 67\,500$, $\bar{v} = 0{\cdot}734$, we obtain $\beta = 2{\cdot}05 \times 10^6$, a value lower than the permissible range of β values. Tanford & Buzzell (1954), using slightly different data, obtained an even lower β value. It seems unlikely that the data used here will be modified sufficiently to give $\beta \geqslant 2{\cdot}12 \times 10^6$, and an explanation of the lower observed value is of importance. Tanford & Buzzell suggested that departure from ellipsoidal form might be a possible explanation. In view of the straightforward use of the Simha viscosity equation and the Perrin expression (connecting frictional and axial ratios for the hydrodynamically equivalent ellipsoid of rotation) in determining the β term, an alternative possibility would be that one (or both equations) is not holding accurately for nearly spherical particles. In particular, for axial ratios smaller than 20, an approximate form of the Simha equation has to be used. For the protein at I 0·1, pH 1·9, we use $[S^0_{20}] = 3{\cdot}46$S, $[\eta] = 0{\cdot}097$ (from Yang & Foster, 1954) and other data as for the neutral protein, which gives $\beta = 2{\cdot}18 \times 10^6$. With normal interpretation this corresponds to an effective elongated ellipsoid of revolution of axial ratio 3·5. The corresponding values of V_e, the effective hydrodynamic ellipsoidal volume, at I 0·1 under neutral and acidic (pH 1·9) conditions are 1·4 and $2{\cdot}7 \times 10^{-19}$ ml. In calculating the former, a shape factor, ν, for viscosity of 3 was assumed. Both values are considerably in excess of

$$M\bar{v}/N = 0{\cdot}82 \times 10^{-19}\ \text{ml.},$$

and an expansion of the molecule at low pH seems clear. However, in view of the failure of the β-term treatment for the protein under neutral conditions, the results of these calculations are to be treated with caution. The data of Miller & Price (1946) for southern-bean mosaic virus, whose molecule appears accurately spherical from electron microscopy, give $\beta = 2{\cdot}3 \times 10^6$, which indicates an axial ratio of 7. On the other hand, recent results on tobacco mosaic virus, which from electron microscopy has a length:width ratio of about 19 in the unaggregated form, give $\beta = 2{\cdot}13 \times 10^6$ and an indicated axial ratio near unity. The data $[S^0_{20}] = 194$S (W. F. Harrington & H. K. Schachman, unpublished results), $[\eta] = 0{\cdot}28$ (Schachman & Kauzmann, 1949), $\bar{v} = 0{\cdot}73$ (Schachman & Lauffer, 1949), and $M = 49 \times 10^6$ (Williams, Backus & Steere, 1951) were assumed. The effective ellipsoid given by the β treatment thus appears to deviate substantially from the particles as they are indicated by electron microscopy, and the difference seems much larger than could be accounted for in terms of bound water.

If 30% hydration of bovine serum albumin is assumed, then the frictional ratios of Table 5 give prolate axial ratios of approximately 4 and 9 for the protein at I 0·1 under neutral and acid (pH 2·3) conditions respectively. At pH 1·9 and lower ionic strengths, even higher frictional ratios occur, a limiting value for zero ionic strength of 1·9 being approached. However, the possible occurrence of primary charge effects at these low ionic strengths makes such extrapolation uncertain. On either derivation of molecular asymmetry, elongation of the molecule occurs at I 0·1 as the pH is lowered and, though the use of higher ionic strengths partially reverses the elongation (as shown by the

S^0_{20}–ionic strength curve of Fig. 13, the limiting frictional ratio at $I \geqslant 0{\cdot}035$ has the value 1·62, considerably in excess of that under neutral conditions. Similar incomplete reversals in viscosity and optical rotation with increasing ionic strength were observed by Yang & Foster (1954). Even so, there are grounds for considering the change to be completely reversed on returning the pH to near neutrality. In particular, J. R. Marrack (personal communication) showed that bovine serum albumin exposed to pH 2 for 1 hr. gave a normal precipitin reaction after neutralization, and Yang & Foster (1954) showed the complete reversal, with increasing pH, of intrinsic viscosity, optical rotation and the binding of anionic detergent. Thus the limited nature of the changes in the protein at pH 1·9 is indicated, a view which is substantiated by the typically native λ_c value obtained under these conditions from optical-rotation measurements. The high temperature coefficient at pH 1·9 is further evidence for the retention of appreciable secondary structure.

Interpretation of polarization data

Against this background of knowledge of the molecule of bovine serum albumin derived from largely established techniques, it is now possible to attempt an interpretation of the polarization data. In the first place the uncorrected values of ρ_h obtained in this work both in neutral solution and at pH 1·9 are in reasonable agreement with those of Weber (1953*a*). Thus only his interpretation of results is questioned.

Further when Weber's fluorescent label has been replaced by a completely different structure (β-anthrylcarbamido-) and suitable correction (on the basis of absorption and fluorescence spectral measurements) for variation in the lifetime of the excited state has been applied, a similar reduction in ρ_h is observed in acid solution and the actual ρ_h values observed are in reasonable agreement with those (corrected) for the naphthyl conjugates. Nor is there any theoretical reason for considering that with lowering of pH similar fluorescence changes would occur for the two unrelated conjugating groups and thus account for depolarization results. The absence of such changes with naphthyl conjugates of ovalbumin again directs attention to possible changes in the protein part of the bovine serum albumin conjugate. The actual ρ_h changes cannot be directly attributed to the increasing molecular asymmetry demonstrated already by sedimentation, diffusion and viscosity measurements, for an increase in relaxation time would thus be expected. Rather it would seem that additional rotational degrees of freedom become available at acid pH values, probably as a result of the high positive charge on the molecule and its consequent swelling. An isotropic swelling was suggested by Yang & Foster, but, as already shown, this is not in accord with physical measurements.

Consideration of some of the established properties of the bovine serum albumin molecule is relevant when the question of intramolecular rotation is discussed. Thus Kharush's (1950) suggestion of 'configurational adaptability' of the molecule, based upon the versatility of its binding properties, and the rapid and easy reversal of denaturation changes in the presence of urea, demonstrated by Kauzmann & Simpson (1953), Simpson & Kauzmann (1953) and Frendsdorf, Watson & Kauzmann (1953), are indications of 'looseness' of structure in the native protein, by which considerable alterations in internal structure would be expected as a result of somewhat minor changes in environment. The high optical rotation ($[\alpha]_D - 60°$) of bovine serum albumin compared with that of most native proteins ($[\alpha]_D - 20°$ to $-40°$) may well be attributable to incompleteness in internal hydrogen bonding which could allow such structural looseness. Thus the occurrence of intramolecular rotation when the equilibrium configuration of the native molecule of bovine serum albumin is disturbed by additional forces arising from the titration of the carboxyl groups seems reasonable. In ovalbumin and other proteins, further intramolecular rotations become possible only when much more drastic alteration of the original structure has occurred.

A decision on the type of rotation which becomes free at acid pH values is not easy. However, certain possibilities are worth mentioning. The limiting relaxation time observed at pH 1·9 ($0{\cdot}60 \times 10^{-7}$ sec.) corresponds with that of a free, compact and reasonably symmetrical molecule of molecular weight 35 000. Weber (1953*b*) has shown that when such units become flexibly joined the change in the observed relaxation time may be slight. Thus it seems possible that equal subunits may occur in the molecule of bovine serum albumin, which under neutral conditions are held by secondary forces so that their relative rotation is not possible. With increasing swelling and distortion of the molecule, such units would be freed, their relative rotation would be possible and their 'free' relaxation times would be observed, without necessarily affecting severely the internal structure of the subunits. A similar explanation seems very likely for fumarase in 0·1 M thiocyanate solution, where the relaxation time observed before dissociation occurs corresponds with that of the dissociation product (P. Johnson & V. Massey, unpublished).

It must be remembered that the molecule of bovine serum albumin has 16–18 intramolecular

cystine residues (Edsall, 1947; Hughes, Saroff & Carney, 1949), which considerably restrain the unfolding of the polypeptide chains. Thus the essential structure within each subunit could be retained by the presence of intramolecular disulphide cross-linkages, but free rotation would be possible about a short peptide segment connecting the two units. Such a hypothesis would be in keeping with the physical properties of bovine serum albumin described, and would explain the low relaxation time observed in acid solution. However, though such a model is likely to represent the truth, other possibilities exist. It would be difficult to rule out completely the freeing at acid pH values of segments of the polypeptide chain whose rotations might suffer various degrees of hindering. However, no reduction in ρ_h for ovalbumin at acid pH appears to occur (Weber, 1952*a*). On either view, with increasing suppression of the electrostatic repulsions at higher ionic strengths, various degrees of hindering would occur, resulting in larger ρ_h values. The opposite trend with increasing temperature is readily explained in terms of increased freedom of rotation with increasing thermal energy.

The indication from optical-rotation measurements of the 'native' type of structure in the molecule of bovine serum albumin at acid pH values is of considerable interest in view of the similar though lower relaxation time occurring in 7 M urea solution, where few hydrogen bonds can remain. Presumably the stabilizing effect of the S–S bridges, limiting the extent of the structural changes, is responsible for this, as it is for the easy (though not necessarily complete) reversal of denaturation by urea and by acids. This view would be in keeping with either the subunit or the more general interpretation of the polarization changes outlined above.

SUMMARY

1. New measurements of the depolarization of the fluorescence of conjugates of bovine serum albumin with fluorescent dyestuffs have been carried out over a range of pH and ionic strengths. Parallel investigations of the light absorption of the conjugating molecule, of the total fluorescence intensity and of the fluorescence spectra were made in order to detect changes in the life time of fluorescence. When such changes are allowed for, a decrease in the rotational relaxation time with decrease in pH below 4 was confirmed.

2. Light-scattering measurements have shown that, down to pH 1·9, over a range of ionic strengths, no change in molecular weight occurs. Sedimentation velocity measurements, with diffusion data already published, confirm this conclusion, but also indicate a swelling and increasing asymmetry of the molecule as the pH is lowered from 4 to 2. Increase in the ionic strength at constant pH only partially reverses such processes. Changes in viscosity, optical rotation and heat content of the solution occur over a similar pH range.

3. The decrease in rotational relaxation time is not directly connected with the increased molecular asymmetry at acid pH values, but must be attributed to increasing freedom of rotational motion within the molecule. Possible models in explanation of the observations are suggested.

4. New sedimentation data for the protein under neutral conditions are reported, and a molecular weight of 67 500, in good agreement with estimates from other methods, is suggested.

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Cholinesterase Inactivation by Snake Venoms

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Although the instability of the cholinesterase of cobra venom in aqueous solution has been pointed out by previous authors (Mounter, 1951; Augustinsson & Grahn, 1952), its cause is unknown. During studies of the cholinesterase of Formosan snake venoms, a high potency of cholinesterase inactivation (anticholinesterase) was found in the cholinesterase-containing venom of the Formosan cobra (*Naja naja atra*) (Chang & Lee, 1955). The existence of such anticholinesterase activity was proved by adding the cobra venom to the venom of *Bungarus multicinctus*, a very potent source of specific (true, acetyl-, aceto-) cholinesterase. Furthermore, in the presence of $MgCl_2$ (0·04 M) its anticholinesterase activity was completely inhibited.

The present work was undertaken to throw more light on the nature of the anticholinesterase activity of the cobra venom and to ascertain whether it can affect cholinesterase preparations from other sources. The results were presented in abstract at the meeting of the American Association for the Advancement of Science, Berkeley, California, in December, 1954.

EXPERIMENTAL

Measurement of cholinesterase activity. The electrometric-titration method described by Alles & Hawes (1940) was adopted in most experiments for the measurement of cholinesterase activity with the aid of a Beckman pH meter, model G, unless otherwise stated. To 14 ml. of 0·0032 M acetylcholine chloride in 0·04 M-$MgCl_2$, 1 ml. of test solution was added. The reaction mixture was kept constantly at 37° by circulating water from a water bath. The pH was kept at 7·4, and the mixture was titrated with 0·02 N-NaOH to neutralize the liberated acetic acid. Cholinesterase activity is expressed in ml. of 0·02 N-NaOH consumed during the first 5 min. of enzymic hydrolysis of substrate.